REPORTS

Cure of *Helicobacter pylori* Infection and Duration of Remission of Low-Grade Gastric Mucosa-Associated Lymphoid Tissue Lymphoma

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Background: **Low-grade B-cell lymphomas arising in mucosa-associated lymphoid tissue (MALT) are most frequently localized in the gastrointestinal tract. More than 90% of gastric MALT lymphomas are diagnosed in patients with chronic, *Helicobacter pylori*-associated gastritis. High remission rates for these lymphomas have been observed after the cure of *H. pylori* infection. Data are lacking, however, with regard to the duration of the remissions. To address this question of remission duration, we have followed 50 patients in whom *H. pylori* infections were eradicated, and we determined whether the patients in complete remission displayed evidence of residual monoclonal B cells during follow-up.** ***Methods:*** **Patients were treated with amoxycillin and omeprazole for 2 weeks in an attempt to cure *H. pylori* infections. Follow-up included endoscopic investigations with biopsy sampling. Monoclonal B cells in biopsy specimens were detected by means of a polymerase chain reaction (PCR)-based assay.** ***Results:*** ***H. pylori*** **infections were cured in all 50 patients. The median follow-up for the 50 patients is currently 24 months (729 days; range, 135–1411 days). Forty patients achieved complete remission of their lymphomas, but five have subsequently relapsed. The median time of continuous complete remission for the 40 patients was 15.4 months (468 days; range, 0–1198 days). Among six patients whose lymphomas did not respond to *H. pylori* eradication, four revealed high-grade lymphomas upon surgery. PCR indicated the presence of monoclonal B cells during follow-up in 22 of 31 assessable patients in complete remission.** ***Conclusions:*** **Complete remissions of low-grade gastric MALT lymphomas after the cure of *H. pylori* infection appear to be stable, although most patients display evidence of monoclonal B cells during follow-up. Whether these patients are truly cured of their lymphomas remains to be determined. [J Natl Cancer Inst 1997;89: 1350–5]**

The concept of mucosa-associated lymphoid tissue (MALT) was introduced by Isaacson and Wright in 1983 *(1).* Until that time, lymphomas arising from MALT were not considered special with regard to biology or clinical behavior. The revised European–American classification of lymphoid neoplasms *(2)* designates MALT lymphomas as ''marginal zone B-cell lymphomas,'' since MALT lymphomas originate from B cells of the marginal zone. MALT lymphomas are most frequently localized in the gastrointestinal tract, and more than 90% of all gastric MALT lymphomas have been shown to be associated with chronic, *Helicobacter pylori*-associated gastritis *(3–5).* Recent epidemiologic, clinical, and molecular biologic evidence has implicated *H. pylori* as an important player in the genesis of gastric MALT lymphomas.

Although clinical studies *(6–8)* have reported high remission rates for low-grade gastric MALT lymphomas after the cure of *H. pylori* infection, data are lacking with regard to the duration of these remissions. We have previously reported *(7)* on 33 patients with stage EI low-grade gastric MALT lymphoma who had been treated using a dual therapy consisting of omeprazole and amoxycillin. We describe herein an extended analysis of 50 total patients (i.e., the initial 33 patients and 17 additional patients) who have been followed for a median time of 24 months. We have addressed the question of whether the remissions induced by *H. pylori* eradication are stable. In addition, we have investigated whether the patients in complete remission after the cure of this infection show a disappearance of clonal B cells, as determined by a loss of monoclonal amplification products (i.e., monoclonal bands) in a polymerase chain reaction (PCR) analysis.

Patients, Materials, and Methods

Patients

Fifty patients with stage EI low-grade gastric MALT lymphomas were included in this prospective multicenter study *(7).* Recruitment was as follows: whenever a gastric biopsy specimen referred to our central pathologist (M. Stolte) revealed the presence of low-grade gastric MALT lymphoma, the referring physician was informed about the study protocol and asked to enroll the patient in the study. Staging included a clinical examination, endosonography, computer tomography of the abdomen and thorax, and a bone marrow biopsy. Only patients with clinical and ultrasound stage EI disease were included *(9).* When the referring physician and the patient agreed to participate, a panel of biopsy specimens was taken at a second endoscopic investiga-

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See ''Notes'' following ''References.''

tion for histologic, microbiologic, and molecular studies.

Thereafter, the patients were treated with a 2-week course of amoxycillin (3 × 750 mg daily) and omeprazole (3 × 40 mg daily). Four weeks after completing this therapy, the first endoscopic control investigations were performed, and these investigations were repeated monthly whenever the patients did not show a regression of their lymphoma. After achieving complete remission, the patients were examined every 6 months. A re-staging was performed every year. Patients not achieving a complete remission were considered to have failed to respond to *H. pylori* eradication and were referred to alternative treatment strategies at week 22 after the completion of eradication therapy. Patients exhibiting no response at the second post-treatment control endoscopy were also categorized as treatment failures and were referred to alternative treatments. The clinical protocol for this study was approved by the local ethical commitees of the Universität Erlangen: Nuremberg and the Virchow-Klinikum, Humboldt-Universität Berlin.

Pathologic Analysis

Pathologic analysis of the biopsy samples was performed as described previously *(7)*. The criteria for the diagnosis of low-grade gastric MALT lymphomas were as follows: unequivocal evidence of lymphoepithelial destruction and replacement of the gastric glands by uniform centrocyte-like cells *(10)*. A judgment of complete histologic regression was rendered when no remnant lymphoma cells could be detected in the post-treatment biopsy specimens and an ''empty'' tunica propria with small basal clusters of lymphocytes and scattered plasma cells was found instead. Partial histologic regression was defined by the presence of post-treatment biopsy samples exhibiting only partial depletion of atypical lymphoid cells from the tunica propria or focal lymphoepithelial destruction.

Detection of Monoclonal B cells by Means of PCR Amplification of Rearranged Immunoglobulin Heavy-Chain Variable (V_H)-Region Genes

PCR was performed with consensus primers essentially as described previously *(7)*, except that a dilution of 1:100 was used for the seminested step instead of a dilution of 1:1000, making the technique more sensitive for monoclonal B-cell detection. Dilution experiments with the B-cell line LAM revealed a sensitivity of 1%–2% for this assay (data not shown) *(11)*. Since we were dealing with biopsy specimens, it was important to control for sufficient amounts of DNA in the amplification reaction mixtures. Such control was achieved by amplification of the β-interferon gene *(12)*. Only samples showing amplification of β-interferon gene sequences were processed further.

To confirm clonality, monoclonal bands were eluted from preparative electrophoretic gels and cloned into TA-vectors (Invitrogen, Leek, The Netherlands) for DNA sequencing. Sequence information was obtained for at least eight clones per sample by means of dye-terminator cycle-sequencing reactions and use of an automated DNA-sequencing machine, following protocols provided by the manufacturer (Applied Biosystems, Foster City, CA). The software package Lasergene (DNA-Star, Madison, WI) was used to align DNA sequences.

Statistical Analysis

Statistical analysis was performed by use of StatView (Abacus Concepts, Berkeley, CA) software for personal computers. The Kaplan–Meier method was used to analyze survival and the duration of remission. In the analysis of complete remission duration, data were censored whenever patients were in continuous complete remission at the last visit that included a gastroscopy. Two patients died in complete remission from causes unrelated to lymphoma (myocardial infarction, n = 1; arterial embolism). The deaths of these two patients were considered as ''events'' in this analysis.

Results

Response to Cure of *H. pylori* Infection

Cure of *H. pylori* infection was obtained in all 50 patients; in two patients, a second treatment course consisting of metronidazole (800 mg/day), clarythromycin (500 mg/day), and omeprazole (40 mg/day) for 7 days was necessary to cure the infection. In one patient, local relapse of MALT lymphoma was associated with reinfection. This reinfection was treated with the triple therapy described above, and the patient is now in second remission. Forty (80%) of the 50 patients went into complete macroscopic and histologic remission, four went into partial remission, and in six, no change was seen after the cure of *H. pylori* infection. Table 1 shows the demographic and response data for all 50 patients.

Follow-up of the 50 Patients

Median follow-up for all 50 patients is now 24 months (729 days; range, 135–1411 days). We first analyzed how stable the complete remissions were. Fig. 1 displays a Kaplan–Meier analysis of the duration of continuous complete remission calculated from the first day that complete remission was achieved. Among the 40 pa-

Table 1. Clinical, histologic, and demographic data on 50 patients with low-grade gastric MALT lymphomas who were treated to eradicate infection with *Helicobacter pylori**,†

	Complete remission (n = 40)			Partial remission (n = 4)			No change (n = 6)		
	Previous study	New	Total	Previous study	New	Total	Previous study	New	Total
No. (%)	24‡ (73)	16 (94)	40 (80)	3‡ (9)	1 (6)	4 (8)	6 (18)	0 (0)	6 (12)
Female/male	11/13	7/9	18/22	2/1	0/1	2/2	2/4	0	2/4
Median age, y (range)	57.5 (31–74)	67.5 (39–77)	61 (31–77)	37 (34–84)	60	48.5 (34–84)	47.5 (35–78)	0	47.5 (35–78)
Tumor stage (by histology)§									
EI	24	16	40	3	0	3	3	0	3
⩾EI	0	0	0	0	1	1	3	0	3
Endoscopic appearance									
Tumor	14	9	23	2	0	2	2	0	2
Ulcer	7	6	13	0	1	1	2	0	2
Mucosal erosion	1	0	1	0	0	0	0	0	0
Atypical mucosa	2	1	3	1	0	1	2	0	2
Tumor size, cm (range)	2.5 (1–10)	3 (1–8)	3 (1–10)	4 (2–5)	1	3 (1–5)	5 (2–8)	0	5 (2–8)

*The 50 patients in this study include 33 patients who were described previously *(7)* and 17 new patients.

†MALT = mucosa-associated lymphoid tissue.

‡One patient in the group previously described was reported as having a partial remission, but this patient developed a complete remission after 17 months of follow-up.

§*See (9)* for information on staging system.

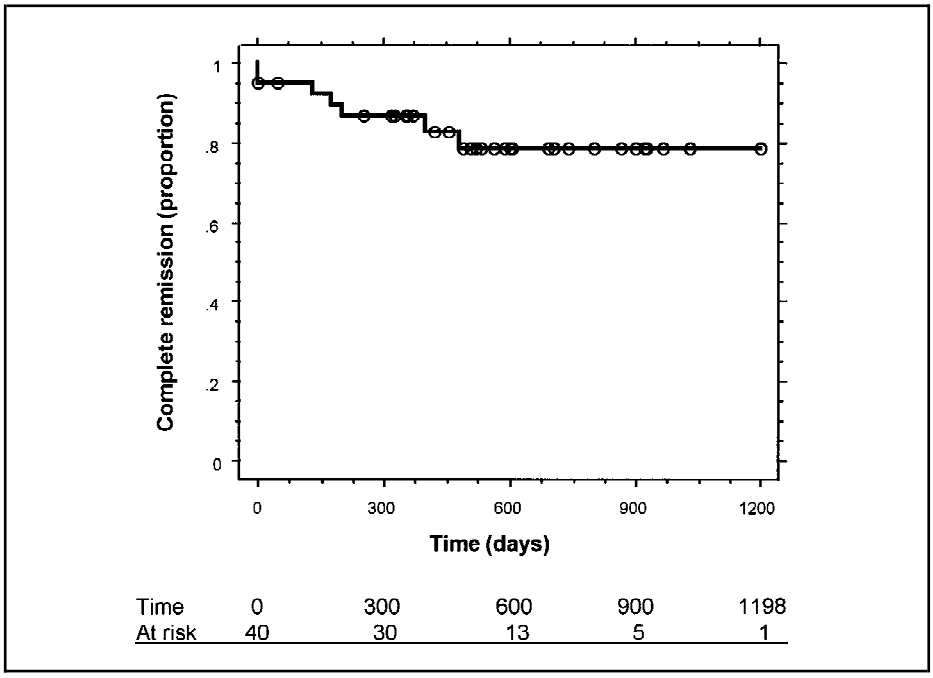

Fig. 1. Duration of complete remission in 40 patients with low-grade gastric MALT (mucosa-associated lymphoid tissue) lymphomas who achieved complete remission after eradication of *Helicobacter pylori* infection. Remission duration was calculated from the first day of complete remission. Four local relapses and one distant lymphoma have been observed thus far. In addition, two patients died in complete remission, and these deaths were treated as ''events'' in this analysis. Continuous complete remission was calculated by use of the Kaplan–Meier method. Censoring events are displayed as circles. The numbers of patients at risk at given time points (days) are displayed below the plot.

tients who achieved complete remission, four local relapses, which were of low-grade, have been observed (174, 198, 399, and 481 days after reaching complete remission). A fifth patient developed a high-grade lymphoma in the nasal cavity (128 days after achieving complete remission). All but one of the patients with a local relapse were *H. pylori* negative at the time of relapse. The median time of continuous complete remission for the 40 patients was 15.4 months (468 days; range, 0–1198 days). No failures have been noted after a continuous complete remission of 500 days, with nine patients being followed for more than 2 years without relapse (Fig. 1). Within the group of 40 patients in complete remission, two have died of cardiovascular causes (53 and 68 years of age).

We also addressed the question of whether the high-grade nasal lymphoma was clonally related to the original MALT lymphoma of the stomach. DNA sequence analysis of the CDR3 region in the rearranged immunoglobulin heavy-chain genes demonstrated that the high-grade lymphoma was not related to the original low-grade gastric MALT lymphoma (Fig. 2).

In regard to the four patients with partial remissions, three were finally referred for gastrectomies. In all three patients, histologic analysis revealed low-grade lymphoma. These patients have been followed for 307, 490, and 1137 days, and no relapse has been observed thus far. The fourth patient (84 years old) died of a stroke while in partial remission of her lymphoma.

Among the six patients not responding to the cure of *H. pylori* infection, four were referred for surgery (gastrectomy, n = 3; subtotal resection, n = 1). In all four patients, histologic analysis revealed high-grade lymphoma in deeper mucosal areas. These high-grade components had not been previously noted in gastric biopsy specimens. Immunohistochemical analysis showed a high-grade B-cell lym-

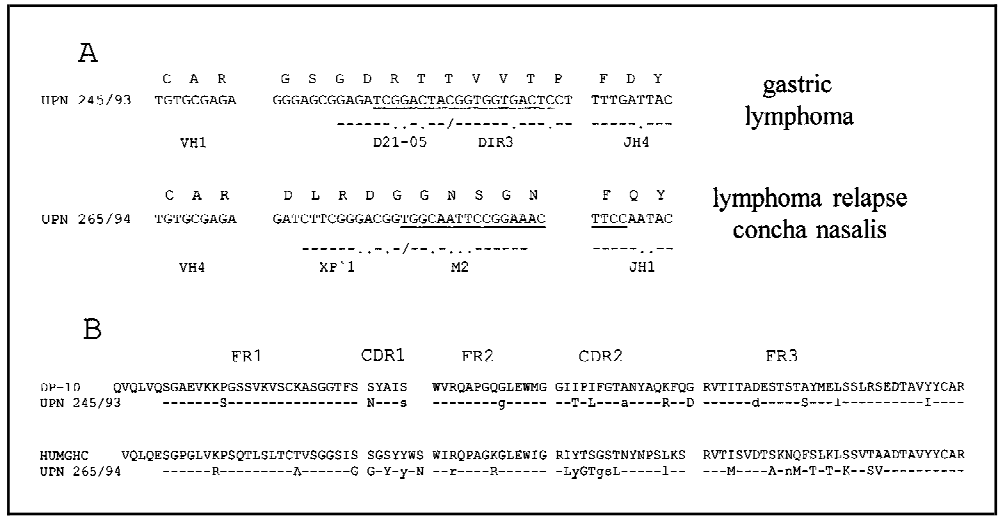

Fig. 2. Comparison of rearranged immunoglobulin heavy-chain variable-region (V_H) gene sequences obtained from the primary gastric low-grade MALT (mucosa-associated lymphoid tissue) lymphoma and a high-grade lymphoma of the nasal cavity (lymphoma relapse concha nasalis), which was found during follow-up, of a single patient. Monoclonal polymerase chain reaction (PCR) products spanning the CDR3 region were cloned and sequenced as outlined in the ''Patients, Materials, and Methods'' section. In **A**), DNA sequences are compared with the published sequences of the germline D and J_H genes. Sequence identity is indicated by dashes; nucleotides that differ from the germline sequences are indicated by dots. As shown, the gastric lymphoma was derived from a D21–05-DIR3-J_H4 rearrangement, whereas the lymphoma of the nasal cavity showed highest homology with an XP'1-M2-J_H1 rearrangement. To study V_H-chain gene usage, clone-specific oligonucleotides were generated on the basis of the unique CDR3 rearrangements (underlined in A) and used in PCR with a consensus primer for framework region 1 (FR1) *(23)*. In **B**), deduced amino acid sequences from the V_H-chain gene sequences are compared with the published sequences of germline V_H alleles. Sequence identity is indicated by dashes; amino acid substitutions are shown in uppercase letters; lowercase letters indicate DNA sequence changes that do not result in an amino acid replacement. In the gastric lymphoma, a V_H1 (DP-10) rearrangement was found, whereas the lymphoma of the nasal cavity contained a V_H4-family allele (HUMIGHC). In the gastric biopsy specimens, no amplification product was generated with the clone specific oligonucleotide for the high-grade lymphoma.

phoma in three of the patients and a high-grade T-cell lymphoma in one. In three of the patients, surgery revealed stage EII disease. Two of the patients referred for surgery died (3 and 5.5 months after surgery), whereas the remaining two are in continuous complete remission. One of the six patients not responding to the cure of *H. pylori* infection was treated with five cycles of chemotherapy (CHOP regimen: 750 mg/m^2 cyclophosphamide, 50 mg/m^2 doxorubicin, 2 mg vincristine, and 100 mg oral prednisone for 5 days every 21 days). This patient is in continuous complete remission 618 days after diagnosis. The sixth patient not responding to the cure of *H. pylori* infection refused to receive any further therapy. The microscopic analysis on his gastric specimens still revealed low-grade lymphoma at 31 months after diagnosis. Fig. 3 shows the overall survival for all 50 patients.

Molecular Studies of Immunoglobulin V_H Gene Rearrangements

A molecular analysis could be performed successfully at diagnosis for 48 of the 50 patients. Monoclonal bands (evidence of clonal B cells) were detected in 38 (79%) of the 48 patients.

We wanted to know whether PCR could be used as an assay for ''molecular remission'' in low-grade gastric MALT lymphoma. Among the 40 patients in complete remission, six patients presented with polyclonal bands, 32 presented with monoclonal bands, and two could not be investigated at all. The lack of monoclonal bands in the six patients just mentioned likely reflects technical problems, since it is known that PCR detects monoclonality only in about 80%–90% of the patients with MALT lymphoma *(11)*. Thirty-one of the 32 patients with monoclonal bands at presentation were investigated during follow-up. Twenty-two (71%) of the 31 patients displayed monoclonal bands continuously during follow-up, although microscopic analysis did not reveal any evidence of remaining lymphoma. The eight patients with the longest molecular follow-up among the 22 patients with ongoing PCR positivity were followed for a median time of 11 months after achieving complete remission (range, 6–23 months). In contrast, nine (29%) of the 31 patients who exhibited monoclonal bands at presentation and were followed molecularly eventually displayed polyclonal states during follow-up.

Discussion

Several clinical studies and case reports have shown that cure of *H. pylori* infection is associated with complete remission in a high proportion of patients with low-grade gastric MALT lymphomas of limited stage. Overall, complete response rates of approximately 80% have been reported *(6–8)*. However, the studies published to date have not described findings on the duration of the remissions. Our study is a follow-up investigation of a study that was published previously *(7)*. For the first 50 patients enrolled in this ongoing study, the median follow-up time is 24 months. As reported in other investigations, complete remissions were obtained in 80% of the patients. Follow-up of the patients in complete remission is in the same range as that for the whole group of patients (23 months).

One question raised in the context of the treatment approach used is whether the cure of *H. pylori* infection yields a cure of this type of MALT lymphoma. Our study shows that most patients who achieve a complete remission do not relapse within a short period of time. However, since low-grade lymphomas have a slow proliferation rate, a follow-up time of 2 years is too short to draw any conclusions as to whether the patients treated with this strategy may indeed be cured of their low-grade lymphomas. A longer follow-up period is needed to answer this question.

Thus far, four local relapses and one distant event have been observed. In the case of the distant lymphoma, a high-grade MALT lymphoma of the nasal cavity was detected, which, by DNA sequence analysis, was not related to the original gastric low-grade MALT lymphoma clone. In the four cases with local relapses, three of the patients were *H. pylori* negative at relapse. Thus, relapses may occur without evidence of reinfection. It has been reported *(13)* that low-grade gastric MALT lymphomas can be observed in *H. pylori*-negative patients. Epidemiologic studies, however, show that low-grade gastric MALT lymphomas have a higher incidence in *H. pylori*-positive patients, and evidence exists that more than 90% of all low-grade gastric MALT lymphomas are observed in a background of *H. pylori*-induced gastritis *(5,14,15)*. How then can one explain the finding that *H. pylori* is probably not necessary for these lymphomas to relapse?

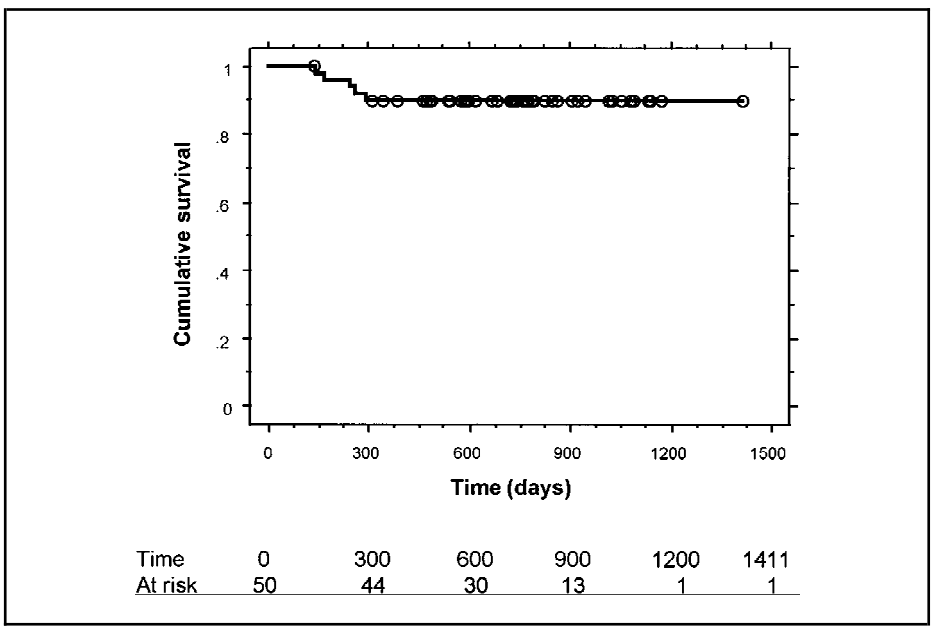

Fig. 3. Cumulative survival analysis for 50 patients with low-grade gastric MALT (mucosa-associated lymphoid tissue) lymphomas who were subjected to a dual antibiotic eradication therapy for infection with *Helicobacter pylori*. A Kaplan–Meier analysis was performed for all 50 patients in the study. Censoring events are indicated by circles. *See* the text and Table 1 for a detailed description of the patients. The numbers of patients at risk at given time points (days) are displayed below the plot.

At the beginning of an early gastric MALT B-cell lymphoma, the tumor cells are still dependent on T-cell helper signals, whereas, later in the process of clonal evolution, T-cell help may not be necessary. Since T cells are needed for early low-grade gastric B-cell lymphoma clones to proliferate *(16,17),* clonal evolution may eventually enable B-cell clones to proliferate without *H. pylori* infection being present. The relapses may thus indicate that the patients had B-cell lymphomas that had evolved from B-cell clones that were already further progressed when the cure of *H. pylori* infection had been performed. Host factors may also play a crucial role in the progression of individual B-cell clones. This possibility is suggested by data from sequencing experiments indicating that, in more than 80% of all gastric MALT lymphomas, V_H alleles known from autoimmune states were used by the lymphoma cells (Thiede C, Alpen B, Bayerdörffer E, Schmidt M, Morgner A, Ritter M, et al.: unpublished results). *H. pylori* may thus drive an autoimmune process in a responsive host that finally results in the evolution of a low-grade MALT lymphoma. At an early stage, the lymphoma may need T-cell signals to survive; however, once genetic damage has progressed to a certain stage, eradication of *H. pylori* may be without a clinical effect.

Eradication of the T-cell stimulus, i.e., *H. pylori,* could therefore be seen as a ''switch'' to indicate how far the B-cell clonal evolution has progressed. This possibility is further underscored by other data obtained from our study: In the patients in whom no response was seen after the cure of the *H. pylori* infection, four patients were referred for surgery. In all four patients, a high-grade lymphoma was detected in deeper mucosal areas. The high-grade lymphoma had not been previously detected in the gastric biopsy specimens. This observation also supports the idea that a clonal evolution may take place; in the beginning, the low-grade B-cell clone is still dependent on T-cell help (and thus susceptible to eradication therapy); then, a low-grade B-cell clone evolves that may not depend on T-cell help; and, finally, a high-grade B-cell clone can be found. In keeping with this scenario, we and others *(18,19)* have recently found that, in some patients with *H. pylori* infection, monoclonal B cells may be detected in the stomach in the absence of any pathohistologic signs of MALT lymphoma. In conclusion, gastric MALT lymphomas may be seen as another model system for tumor evolution in addition to colon cancer or chronic myelogenous leukemia *(20–22).*

One observation, which has also been made by others, is that the remission of low-grade gastric MALT lymphomas may take some time after the cure of *H. pylori* infection, unlike the situation with chemotherapy or radiotherapy. This finding is consistent with the idea that these B-cell lymphomas must get a ''death'' signal that is probably mediated by other cells, e.g., specific T cells responding to bacterial antigens/superantigens *(16,17).* In our study, several patients achieved complete remission after a period of 6 months. However, the median time to reach a complete remission from the start of therapy was 5.5 months. It is important to note that even the patients who achieved a complete remission very late showed some signs of regression, either endoscopically or histologically, before complete remission was obtained. Patients not showing any signs of regression may, as indicated by the four patients referred for surgery, harbor high-grade lymphomas. How should patients with low-grade gastric MALT lymphomas be treated? One approach would be first to cure the patients with stage EI low-grade gastric MALT lymphomas of *H. pylori* infection and then to examine them every 4 weeks. In the event that no response is seen after two or three consecutive examinations, either radiotherapy or surgery would be indicated. Whenever there is evidence for transformation into high-grade lymphomas, chemotherapy (e.g., the CHOP regimen) may be given. Since eradication treatment of *H. pylori* infection should still be considered as investigational, this kind of therapy should not be performed outside of clinical trials.

In addition to clinical and histologic follow-up, we also performed molecular studies of the rearranged V_H-gene sequences by means of PCR. These investigations showed that, using this sensitive approach for monoclonal B-cell detection, most patients remained monoclonal after the cure of *H. pylori* infection and complete remission of the lymphoma. It is unclear at the present time whether the B cells giving rise to the monoclonal bands represent clonal cells belonging to the ''malignant'' clone or whether they may be so-called ''memory'' B cells. We are currently investigating this question by means of more detailed molecular studies. Since the ''gold'' standard in the management of these lymphomas is still histology, we do not know what a monoclonal PCR result really means. Do the patients with monoclonal PCR bands harbor a higher probability of having a relapse of their disease? Three of the four patients with a local relapse, who were monoclonal at diagnosis, were monoclonal before the relapse was detected. At present, however, PCR cannot replace histology, since in other studies *(18,19)* no lymphomas have been detected, even though PCR indicated the presence of monoclonal cells.

In summary, this study shows that long-lasting remissions can be observed after the cure of *H. pylori* infection in patients with low-grade gastric MALT lymphomas of limited stage. Whether the cure of disease is achieved through use of this novel approach in the treatment of gastric MALT lymphomas, however, is still open to question, since longer follow-up investigations are needed. Thus, infection with *H. pylori* plays an important role in the genesis of this disease, although it may not be the only causal factor.

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Notes

Supported by a grant from the Bayerische Gastroenterologische Gesellschaft, the Wilhelm Sander Stiftung (A. Neubauer), and the Deutsche Forschungsgemeinschaft (A. Neubauer).

We thank the participating physicians for their help throughout the study.

Presented in part at the American Society of Clinical Oncology meeting, May 1996.

Manuscript received April 8, 1997; revised July 11, 1997; accepted July 16, 1997.

Relationship Between the Size and Margin Status of Ductal Carcinoma *In Situ* of the Breast and Residual Disease

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Background: **For women with ductal carcinoma *in situ* (DCIS) of the breast who have been treated with breast-conserving surgery, the usefulness of size and surgical margin status (i.e., presence or absence of disease at the point of excision) as prognostic factors for predicting residual disease has not been well established. This study was conducted to determine more clearly the relationship between size and margin status of mammary DCIS to residual disease.** ***Methods:*** **The pathology records of 232 consecutive patients with mammary DCIS who had been initially treated with lumpectomy at the University Hospitals of Cleveland were retrospectively reviewed. The size of the DCIS and the surgical margins of lumpectomy were analyzed. Residual disease was defined as the persistence of DCIS in the re-excision and/or mastectomy specimens.** ***Results:*** **Residual disease was found in 15 of 101 patients with DCIS of less than 1.0 cm in longest dimension, in 27 of 96 patients with DCIS of 1.0–2.4 cm in size, and in 24 of 35 patients with DCIS of greater than or equal to 2.5 cm in size ($P<.001$). Residual disease was found in 30 of 77 patients with DCIS and positive margins, in 11 of 59 patients with DCIS and close margins (≤1 mm), and in 10 of 73 patients with DCIS and negative margins (>1 mm) ($P = .001$). In multivariate analysis, the occurrence of residual disease was associated with large tumor size (i.e., ≥2.5 cm) (odds ratio [OR] = 7.7; 95% confidence interval [CI] = 3.13–20.00; two-sided $P = .0001$) and with positive margin status (OR = 2.2; 95% CI = 1.02–4.55; two-sided $P = .04$).** ***Conclusions:*** **The size and margin status of DCIS each were found to be independent predictors of residual disease. [J Natl Cancer Inst 1997;89:1356–60]**

Ductal carcinoma *in situ* (DCIS) of the breast is being diagnosed with increasing frequency in recent years, primarily because of early detection efforts and the use of screening mammography. It has been estimated that 23 000 new cases of DCIS are diagnosed annually in the United States *(1)*, accounting for approximately one third of all mammographically detected breast cancers *(2–4)*. Breast preservation has become an increasingly popular treatment option for many patients with breast cancer *(1)*, despite the increased risk of local failure compared with mastectomy *(5–8)*. This situation emphasizes the importance of identifying a subset of breast cancer patients who are at high risk for disease recurrence following conservative therapy, as ablative surgery remains an appropriate therapy for certain patients at high risk for local failure.

Residual disease is an important factor in local failure of treatment/recurrence of disease following conservative therapy for DCIS of the breast *(8,9)*. The ability to predict patients who are at high risk for residual disease/recurrence helps to determine treatment options. The role of biopsy margin status and tumor size as predictors of residual disease/recurrence in invasive breast carcinoma is well established *(9–15)*. However, the relevance of the size of mammary DCIS and the margin status of lumpectomy specimens is uncertain *(16)*.

In this study, we report the relationship between the size of DCIS and the margin status of lumpectomy specimens and residual disease in subsequent re-excision or mastectomy specimens from 232 patients.

Patients and Methods

Patients. Two hundred thirty-two consecutive patients who had been diagnosed with mammary DCIS at University Hospitals of Cleveland and Case Western Reserve University during the period from January 1980 through December 1993 were studied. The criteria for inclusion in the study were as follows: 1) The patient had a mammary DCIS on an excisional biopsy/lumpectomy, 2) the histologic slides were available for review, and 3) follow-up information was available. Clinical information and follow-up data were obtained from medical records and the tumor registry of the Ireland Cancer Center of the University Hospitals of Cleveland. Of the 232 patients, 166 (72%) presented with a mammographic abnormality detected on routine mammograms, 51 (22%) presented with a palpable mass, and the remaining 15 (6%) presented with other symptoms, including, among others, nipple discharge, cosmetic surgery, Paget's disease of the nipple, nipple retraction, and pigmentation. The patients ranged in age from 18 to 87 years (median, 59 years). The ratio of blacks to whites was 1:3.7. Patient follow-up ranged from 3 months to 171 months (median, 45 months). All patients initially underwent lumpectomy. Ninety patients (39%) subsequently underwent mastectomy, and 142 patients (61%) were treated with breast-conserving surgery (lumpectomy with or without re-excision). Among the 142 patients who were treated with breast-conserving surgery, 58 (41%) had re-excision, 16 (11%) received radiation therapy following the initial lumpectomy, and the remaining 68 (48%) did not receive any other treatment (lumpectomy only).

Histologic evaluation. To process the tissue, we painted the entire external surface of the specimen with India ink and then serially sectioned the tissue perpendicular to the long axis of the specimen at 3-mm intervals. The sections were fixed in 10% formalin, embedded in paraffin, cut at 3 μm, and stained with hematoxylin–eosin. The specimens ranged from 1.3 to 18 cm in largest dimension (median size, 6.0 cm). Of the 232 specimens, 167 (72%) were embedded entirely for histologic examination. A diagram designating the sections submitted for histologic examination was available for 86 biopsy specimens. The mean number of slides reviewed from each biopsy was 14 (range, 2–55; median, 13). The margin status was evaluated microscopically. A positive margin was defined as tumor extending to or transected by the inked margin (Fig. 1, A); a close margin was defined as tumor less than or equal to 1 mm from the inked margins, but not transected by the inked margin (Fig. 1, B); a negative margin was defined as tumor greater than 1 mm from the inked margins. The margin was considered indeterminate when the biopsy specimen was not inked or the tissue was fragmented. The size of DCIS was determined by microscopic measurement of the largest two dimensions from the glass slide, combined with diagrammatic mapping of the lumpectomy specimen. The third dimension of DCIS, in cases where a diagram was not available, was calculated by multiplying the number of sections containing DCIS by the thickness of the section, which was estimated to be 0.3 cm. For those specimens for which the diagrams were available, the third dimension of tumor size was determined by studying the distribution of DCIS in the biopsy specimen by use of the diagrammatic mapping. This technique of size measurement has been used by several staff pathologists in our department since 1987 and yields reproducible re-

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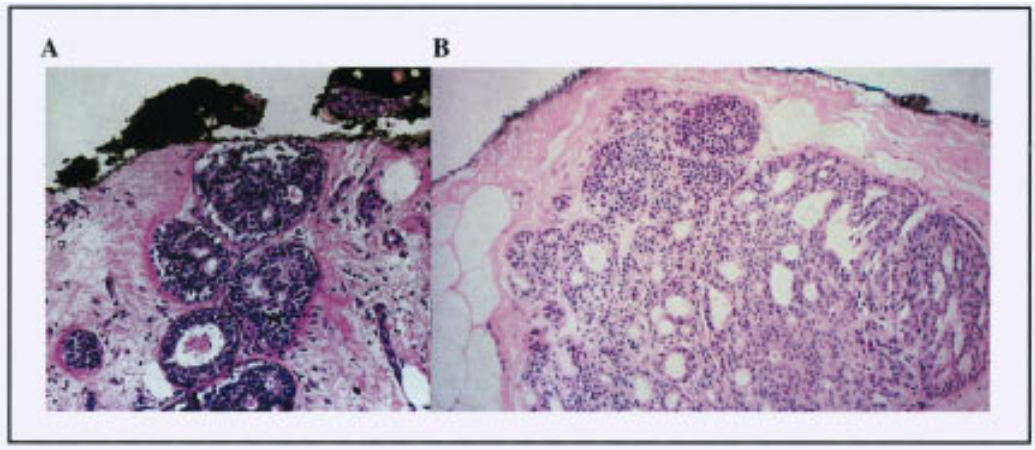

Fig. 1. Histologic evaluation of margin status of breast biopsy specimens of ductal carcinoma *in situ.* **A)** Positive margin, ink on tumor cells. **B)** Close margin, tumor ⩽1 mm from inked margin.

sults with interobserver variability of less than 10%. The largest dimension of DCIS was used in the final analysis. Residual disease was defined as the persistence of DCIS in the re-excision and/or mastectomy specimens.

Statistical analysis. The relationship between residual disease (0 = absent; 1 = present) and several clinicopathologic characteristics was examined. The chi-squared test was utilized to describe the association of residual disease with tumor characteristics. The odds ratio (OR) and its 95% confidence interval (CI) for the relationship of residual disease to the clinical presentation (0 = palpable mass; 1 = mammographic detection), race (0 = white; 1 = black), age, size (0 to <2.5 cm; 1 to ⩾2.5 cm), and margin status (0 = negative or close margins; 1 = positive margins) were also utilized. Significance levels for ORs were evaluated by use of the Wald-*P* chi-squared statistic. An OR with a 95% CI that does not include 1 denotes a statistically significant relationship. Multivariate analysis of the relationship of residual disease and other clinicopathologic features was performed by use of logistic regression.

Results

Residual disease was present in a total of 66 (28%) of 232 cases. Residual DCIS was observed at the previous excision site in the majority of patients for whom these data were available. The mean length of the largest dimension of DCIS was 1.4 cm (range, 0.3–5.4 cm). Residual disease was found in 15 (15%) of 101 DCIS lesions that were less than 1.0 cm in longest dimension, 27 (28%) of 96 lesions that were 1.0–2.4 cm in size, and 24 (69%) of 35 lesions that were greater than or equal to 2.5 cm in size ($P<.001$) (Table 1). The margins were positive in 77 (37%) patients, close in 59 (28%) patients, and negative in 73 (35%) patients. The margin status in the remaining 23 patients (all diagnosed prior to 1985) was indeterminate or unknown. Residual disease was found in 15 (65%) of 23 of these patients. Among patients with known margin status, residual disease was found in 30 (39%) of 77 patients who had DCIS with positive margins, 11 (19%) of 59 patients who had DCIS with close margins, and 10 (14%) of 73 patients who had DCIS with negative margins (Table 1). The increase in the patient's risk of residual disease when the margins were positive for DCIS was statistically significant (overall $P = .001$). The presence of close margins in biopsy specimens was associated with a slightly higher but not a statistically significant increase in risk of residual disease (19% versus 14% for negative margins, $P>.3$). Residual disease increased in frequency with increasing tumor size (Fig. 2, A). In addition, tumor size correlated significantly with margin status (Fig. 2, B). Seventy-seven percent of patients with DCIS of greater than or equal to 2.5 cm in size had positive margins at initial biopsy versus 30% of patients with DCIS of less than 2.5 cm ($P<.001$).

In the multivariate analysis, the finding of residual disease was significantly associated with large size of DCIS ($P = .0001$) and positive margins ($P = .04$). Patients with DCIS of greater than or equal to 2.5 cm in size had an OR of 7.7 (95% CI = 3.13–20.00) for residual disease compared with patients with DCIS of less than 2.5 cm. If we control for the size of DCIS, patients with positive margins in lumpectomy specimens had an OR for risk of residual disease of 2.2 (95% CI = 1.02–4.55) compared with those with negative or close margins. Fig. 3 illustrates that 29 (46%) of 63 patients with DCIS of greater than or equal to 1.0 cm with positive margins had residual disease compared with eight (23%) of 35 patients with DCIS of greater than or equal to 1.0 cm with close margins and five (26%) of 19 patients with DCIS of greater than or equal to 1.0 cm with negative margins. Among patients with small DCIS (<1.0 cm), residual disease was found in two (14%) of 14 patients with positive margins, in two (8%) of 24 patients with close margins, and in three (5%) of 54 patients with negative margins. Age, race (black versus white), and clinical presentations (palpable mass versus mass detected mammographically) were not signifi-

Table 1. Association of histologic size and margin status of mammary ductal carcinoma *in situ* with residual disease

	No. of patients	Residual disease		
		Present	Absent	*P**
Tumor size, cm				
<1.0	101	15 (15%)	86 (85%)	
1.0–2.4	96	27 (28%)	69 (72%)	
⩾2.5	35	24 (69%)	11 (31%)	<.001
Margin status†				
Negative	73	10 (14%)	63 (86%)	
Close	59	11 (19%)	48 (81%)	
Positive	77	30 (39%)	47 (61%)	.001

*Two-sided *P* value was obtained by chi-squared test.

†A positive margin was defined as tumor extending to the inked margins; a close margin is tumor ⩽1 mm from the inked margins; a negative margin is tumor >1 mm from the inked margins.

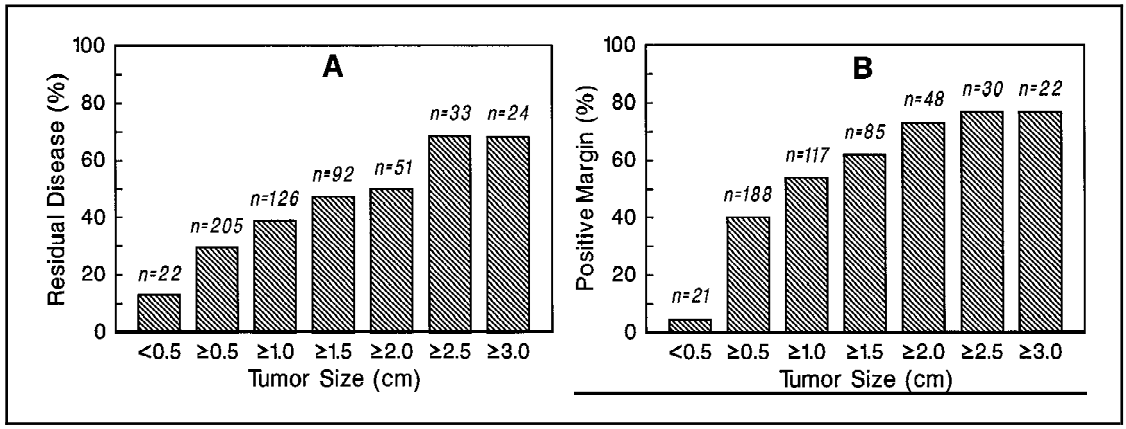

Fig. 2. Association of size of mammary ductal carcinoma *in situ* with residual disease **(A)** and margin status **(B).** Patients with indeterminate margins are not included in **B.** (n = number of cases).

cantly associated with residual disease in the multivariate analysis.

Local recurrence of disease was seen in 10 (7%) of 142 patients during a mean follow-up period of 49 months. All of these 142 patients were initially treated conservatively with lumpectomy with or without re-excision. The mean interval from lumpectomy to recurrence was 77 months (range, 29–165 months; median, 67 months). Four of these patients had positive margins in the lumpectomy specimen, and three had close margins. The tumors ranged in size from 0.4 to 4.0 cm (mean, 2.0 cm). Half of these recurrences were invasive carcinomas. None of the patients who had mastectomy following lumpectomy developed local recurrence. Six patients died during the period of the study (four from lung cancer, one from congestive heart failure, and one from contralateral breast cancer).

Discussion

DCIS of the breast is a heterogeneous group of lesions with a wide spectrum of clinical and pathologic manifestations *(2,17–21)*. The optimal treatment for DCIS is still controversial. The ultimate outcome of breast perservation in the treatment of DCIS is largely unknown. Multiple factors have to be considered when one is deciding upon the optimal treatment for a particular patient with mammary DCIS. It is evident that a subgroup of patients is at higher risk of local failure after breast-conserving surgery. Factors contributing to local failure are important considerations in selecting the appropriate therapeutic approach for mammary DCIS. Additional therapy such as postoperative radiation treatment or mastectomy may benefit patients who are at high risk for local failure.

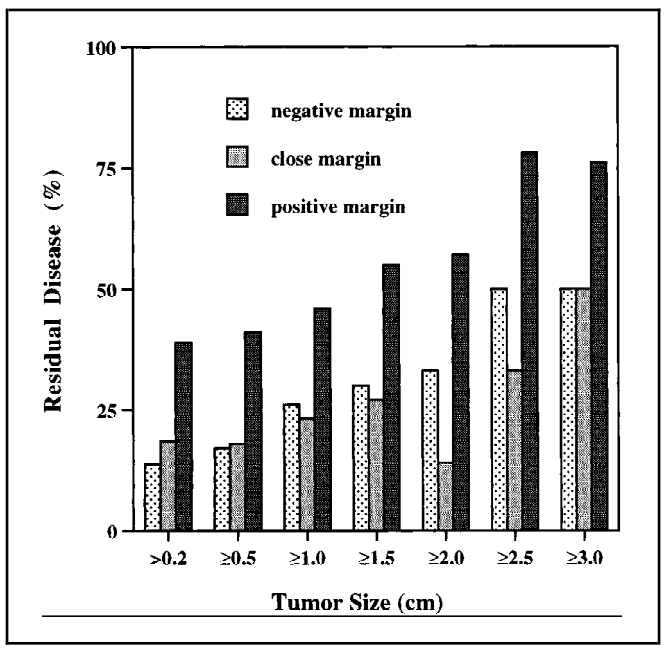

Fig. 3. Relationship between margin status of breast biopsy specimens of ductal carcinoma *in situ* (DCIS) and residual disease in subsequent re-excision/mastectomy specimens after controlling for the size of DCIS.

Three-dimensional imaging of DCIS provides an accurate evaluation of the extent of DCIS in the mammary ducts *(22)*. This technique, however, is difficult to apply to routinely processed surgical specimens. Since most DCIS lesions cannot be identified grossly with certainty, assessment of the extent of DCIS in routinely processed breast biopsy specimens is feasible utilizing the diagrammatic mapping of biopsy specimens. Our findings indicate that measurement of the DCIS size from the histologic slides in conjunction with margin status evaluation provides an accurate assessment of risk of residual disease. Lesions smaller than 1.0 cm in the longest dimension, accounting for 44% (101 of 232) of the patients with mammary DCIS in this study, had no residual DCIS following lumpectomy when the margins were free of DCIS. Patients with DCIS lesions that were greater than or equal to 2.5 cm in longest dimension had the highest risk of residual disease regardless of margin status. Patients with DCIS size between 1.0 and 2.4 cm in largest dimension had 28% risk of residual disease. The ability to predict residual disease is greatly improved by assessing the margin status in these patients. Thirty (39%) of 77 patients with positive margins had local failure versus 11 (19%) of 59 patients with close margins and 10 (14%) of 73 patients with negative margins. After we controlled for the tumor size, patients with positive margins had approximately twice the risk of residual disease than patients with negative or close margins. The risk of residual disease was higher in patients with positive margins than in patients with close margins (39% versus 19%), whereas there was only a marginal increase in risk of residual disease in patients with close mar-

gins compared with those with negative margins. The risk of residual disease in patients with close margins was only slightly higher than that in patients with negative margins (19% versus 14%, $P>.3$). In addition, there was a significant ($P<.0001$) association between the extent of DCIS and positive margin status. At initial biopsy, 77% of large tumors (≥2.5 cm) had positive surgical margins. Because of the small number of patients who received radiation therapy following lumpectomy, the role of adjuvant radiation therapy as a treatment modality cannot be assessed in this study.

Our findings support the important role of margin status assessment in local disease control in patients treated conservatively. Negative margins, however, will not guarantee complete excision of the DCIS, and local recurrence can still occur. A high percentage of patients had local recurrence of disease because of residual disease even when the margins of lumpectomy were clear *(16,23–25)*. Additional risk factors, such as tumor size, should be considered in the assessment of risk of residual disease in patients treated with breast-conserving surgery. Our data indicate that both margin status and size of DCIS are important considerations in stratification of patients for different therapeutic options. The combination of DCIS size and surgical margin status identified a subgroup of patients with increased risk of local failure. More aggressive treatment may be appropriate in such patients. At present, there is general agreement on what constitutes a clear margin for invasive breast carcinomas *(11)*. It is not clear, however, what constitutes a clear surgical margin for DCIS. Our study arbitrarily uses 1 mm as a clear margin for DCIS. Faverly et al. *(22)* have demonstrated skip lesions (i.e., areas of DCIS with intervening normal breast epithelial tissue) in low-grade DCIS. In addition, depending on the plane of sectioning, the distance between any two branches of the same duct involved by DCIS can exceed the distance designated as a clear margin. Therefore, residual DCIS may remain in the patient's breast after surgery even when the "clear margin" exceeds 10 mm. The DCIS observed in the re-excision most likely represents DCIS that was incompletely removed by the initial excision rather than multicentric/multifocal DCIS or recurrent disease. This hypothesis is supported by our findings and the findings of Silverstein et al. *(23)* who reported residual DCIS in the immediate vicinity of the previous biopsy/excision site in the majority of cases. Clearly, as Silverstein et al. *(25)* elegantly demonstrated, histopathologic features such as nuclear grade and presence of comedo-type necrosis are important prognostic factors that can influence the rate of recurrence of DCIS *(25)*. Some evidence exists that these histopathologic features are not independent prognostic factors but rather that they define subsets of DCIS having different histopathologic, clinical, molecular, and biologic features *(9,25)*. Further studies are necessary to clearly characterize these subsets of DCIS.

In summary, the size and the margin status of mammary DCIS are each independent, statistically significant factors predicting local failure. Small DCIS tumors (<1.0 cm) with negative margins carry a low risk of local failure and can be treated conservatively with lumpectomy. Large DCIS tumors (≥2.5 cm) pose a particular risk of residual disease regardless of margin status, and additional adjuvant therapy may be necessary.

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Note

Manuscript received February 10, 1997; revised July 10, 1997; accepted July 17, 1997.

Cancer Incidence in a Population-Based Cohort of Patients Hospitalized With Diabetes Mellitus in Denmark

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Background: **Diabetes has been associated with an increased risk of several cancers, notably cancers of the pancreas, liver, endometrium, and kidney. Since most previous studies have involved a limited sample size or focused on specific cancer sites, we conducted a comprehensive assessment of the risk of cancer in a nationwide cohort of diabetics in Denmark.** ***Methods:*** **Discharge records of 109 581 individuals hospitalized with a diagnosis of diabetes from 1977 through 1989 were linked with national cancer registry records through 1993. Standardized incidence ratios (SIRs) were calculated for specific cancer sites.** ***Results:*** **The SIRs for primary liver cancer were 4.0 (95% confidence interval [CI] = 3.5–4.6) in males and 2.1 (95% CI = 1.6–2.7) in females. These SIRs remained elevated with increasing years of follow-up and after exclusion of patients with reported risk factors (e.g., cirrhosis and hepatitis) or patients whose cancers were diagnosed at autopsy. Kidney cancer risk was also elevated, with SIRs of 1.4 (95% CI = 1.2–1.6) in males and 1.7 (95% CI = 1.4–1.9) in females. For both sexes combined, the SIR for pancreatic cancer was 2.1 (95% CI = 1.9–2.4), with a follow-up time of 1–4 years; this SIR declined to 1.3 (95% CI = 1.1–1.6) after 5–9 years of follow-up. Excess risks were also observed for biliary tract and endometrial cancers. The SIRs for kidney and endometrial cancers declined somewhat after exclusion of diabetics with reported obesity.** ***Conclusions:*** **Patients hospitalized with a diagnosis of diabetes appear to be at higher risk of developing cancers of the liver, biliary tract, pancreas, endometrium, and kidney. The elevated risks of endometrial and kidney cancers, however, may be confounded by obesity. [J Natl Cancer Inst 1997;89:1360–5]**

Diabetes mellitus is a metabolic disease of two major subtypes that is characterized by abnormalities in the synthesis and cellular uptake of insulin, a critical hormonal regulator of glucose metabolism. In insulin-dependent diabetes mellitus (IDDM), insulin synthesis ceases as a result of the autoimmune destruction of insulin-producing pancreatic islet cells, which is thought to be triggered by an environmental factor (i.e., viral infection) primarily in individuals who are positive for the histocompatibility antigens HLA-DR3 and/or HLA-DR4 *(1).* In noninsulin dependent diabetes mellitus (NIDDM), pancreatic islet cells continue to secrete insulin, but target tissues (e.g., muscle and liver) are resistant to its uptake and use because of a decrease in the number of insulin receptors, alterations in postreceptor function, or the presence of blocking antibodies.

Elevated risks have been reported in diabetics for several cancers, notably cancers of the pancreas *(2,3),* liver *(4–8),* endometrium *(5,8),* and kidney *(5,9).* Most previous studies of cancer risk in diabetics have been based on a limited sample size, or they have focused on population subgroups or specific cancer sites. This study provides a comprehensive assessment of multiple cancer sites in a large, population-based cohort of diabetics and was undertaken by linking computerized records from nationwide hospital and cancer registries in Denmark.

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Methods

The cohort was established by identifying all males and females in the Danish Central Hospital Discharge Register who were hospitalized with diabetes as a primary or a secondary diagnosis during the years 1977 through 1989. From 1977 through 1986, these individuals were identified by International Classification of Diseases [ICD]-8 code 250 for diabetes *(10)*, and, from 1987 through 1989, by revised codes from the Danish National Board of Health that distinguished IDDM and NIDDM. The cohort entry date was defined as the first day of the month after the initial hospital discharge in which diabetes was identified.

Of the 117 689 diabetics initially identified, 8106 were excluded from the cohort because they died during the brief interval between hospital admission and the cohort entry date, while an additional two individuals were excluded because of questionable age data, leaving a total of 109 581 diabetics for inclusion in the cohort. Estimates of cancer risk in the cohort exclude the 2222 cancers (and corresponding 97 267 person-years) diagnosed during the first year of follow-up, which were assumed to be prevalent at cohort entry and possibly diagnosed as a result of clinical evaluation for diabetes. However, the subjects with these 2222 cancers were retained in the analysis because they remained at risk of developing another primary cancer and national incidence rates in Denmark include multiple primaries.

To ascertain cancer incidence in the cohort, computerized hospital discharge records were linked to the Danish Cancer Registry by use of a personal identification number assigned to all Danish citizens. The total number of incident cancers observed during the follow-up period was 11 053. The cohort exit date was defined as either the date of death or December 31, 1993. Additional information was obtained from the Hospital Discharge Register on up to 20 medical conditions reported at each admission during the observation period. The Hospital Discharge Register *(11)* and the Cancer Registry *(12)* have reported a completeness of registration of more than 97% for discharges and incidence of cervical cancer.

Site-specific standardized incidence ratios (SIRs) and 95% confidence intervals (CIs) were calculated to compare the observed number of incident cancers with the expected. The number of expected cancers was generated by multiplying the number of person-years in the cohort by the national cancer incidence rates, specified for sex and 5-year-age and calendar year categories. For individuals with multiple primary tumors (including second primaries of the same site), each tumor was counted separately in the analysis. Site-specific SIRs were also stratified on the basis of sex, length of follow-up in years, diabetes type for those entering the cohort from 1987 through 1989, and whether or not the hospital records mentioned obesity, a confounding risk factor for several cancers. Chi-squared tests *(13)* were used to assess trends in risk estimates according to years of follow-up. Primary liver cancer SIRs were further stratified on the basis of the presence or absence of cancer-associated medical conditions, and, along with renal cell cancer, according to the inclusion or exclusion of autopsy-diagnosed cases from both the observed and the expected numbers.

To address concerns of selection bias arising from the use of a hospitalized study population, SIRs were stratified on the basis of diabetes diagnostic order (i.e., whether diabetes was the sole or the primary hospital discharge diagnosis at cohort entry or whether it was a secondary diagnosis). Presumably, SIRs would be higher in the stratum with diabetes as a secondary diagnosis if subjects were preferentially selected into the cohort by virtue of having other hospital diagnoses that predisposed to subsequent cancer.

Results

After exclusion of ineligible subjects and of cancers diagnosed within 1 year of cohort entry, a total of 8831 incident cancers and 628 129 person-years were included in the present analyses. Among the 19 363 cohort members accrued from 1987 through 1989 (17.7% of the total cohort), when diabetes subtypes could be differentiated by diagnostic codes, 15 495 (80%) were assigned a code for NIDDM and 3868 (20%) were assigned a code for IDDM. The overall median age at cohort entry was 64 years in males (n = 54 571) and 69 years in females (n = 55 010), with 4.3% of the cohort entering prior to the age of 20 years. The median age for patients with NIDDM entering the cohort from 1987 through 1989 was 69 years compared with 51 years for patients with IDDM. A total of 56.3% of the cohort died during follow-up.

Sex-specific SIRs for major cancer sites are shown in Table 1. Elevated risks of digestive system cancers were observed in both males and females. These higher risks were primarily due to excess liver, pancreatic, and biliary tract tumors. Most notably, the SIR of primary liver cancer was 4.0 (95% CI = 3.5–4.6) in males and 2.1 (95% CI = 1.6–2.7) in females. Approximately 60% of these liver cancers were hepatocellular carcinoma, 19% were cholangiocarcinoma, and 2.5% were combined hepatocellular and cholangiocarcinoma, while the remainder were primarily miscellaneous rare types (1.5%), unspecified tumors (10%), or tumors that were not histologically confirmed (7%). The histologic distribution of primary liver cancers among diabetics and the percentage of histologically confirmed tumors approximated that of the tumor registry.

Table 1. Standardized incidence ratios (SIRs) of cancer in patients hospitalized with diabetes at cohort entry, stratified according to sex (Denmark, 1977–1989)

Type of cancer (ICD-7 code[s])*	Males			Females		
	No.	SIR	95% CI†	No.	SIR	95% CI
All cancers (140–205)	4666	1.1	1.1–1.1	4165	1.1	1.1–1.1
Mouth and pharynx (140–148)	118	1.2	1.0–1.4	54	1.2	0.9–1.6
Digestive organs (150–159)	1433	1.4	1.3–1.5	1206	1.2	1.2–1.3
Esophagus (150)	67	1.3	1.0–1.6	26	1.0	0.7–1.5
Stomach (151)	188	1.2	1.0–1.3	131	1.1	1.0–1.4
Small intestine (152)	14	1.3	0.7–2.2	12	1.3	0.7–2.2
Colon (153)	413	1.3	1.1–1.4	442	1.1	1.0–1.2
Rectum (154)	235	1.1	0.9–1.2	167	1.0	0.9–1.2
Liver (155.0)	190	4.0	3.5–4.6	68	2.1	1.6–2.7
Biliary tract (155.1–.3)	39	1.4	1.0–1.9	81	1.4	1.1–1.8
Pancreas (157)	206	1.7	1.5–2.0	211	1.6	1.4–1.9
Larynx (161)	61	1.0	0.8–1.3	5	0.5	0.2–1.1
Lung (162)	713	1.0	0.9–1.1	250	0.9	0.8–1.1
Breast (170)	7	1.1	0.4–2.2	777	1.1	1.1–1.2
Ovary (175)	—	—	—	129	0.9	0.7–1.0
Corpus uteri (172)	—	—	—	231	1.4	1.2–1.6
Cervix (171)	—	—	—	92	0.9	0.7–1.1
Other female genital (176)	—	—	—	61	1.5	1.2–2.0
Prostate (177)	505	0.9	0.8–1.0	—	—	—
Testis (178)	23	1.0	0.6–1.5	—	—	—
Kidney (180)	168	1.4	1.2–1.6	154	1.7	1.4–1.9
Bladder (181)	383	1.0	0.9–1.1	110	0.9	0.8–1.1
Melanoma (190)	61	1.0	0.7–1.2	77	1.0	0.8–1.3
Nonmelanoma skin (191)	613	1.0	0.9–1.1	461	0.9	0.8–0.9
Brain, nervous system (193)	80	1.1	0.9–1.4	79	1.1	0.8–1.3
Thyroid (194)	10	1.3	0.6–2.3	21	1.2	0.7–1.8
Endocrine (195)	5	1.4	0.5–3.4	0	0.0	0.0–1.4
Lymphatic and hematopoietic (200–205)	272	1.1	1.0–1.2	239	1.1	1.0–1.3
Lymphoma (200–202)	108	1.1	0.9–1.3	97	1.1	0.9–1.4
Multiple myeloma (203)	48	1.0	0.8–1.4	52	1.3	1.0–1.7
Leukemia (204)	116	1.1	0.9–1.3	90	1.1	0.9–1.4

*ICD-7 = International Classification of Diseases, seventh revision *(43)*.

†95% CI = 95% confidence interval.

The SIR for pancreatic cancer in males (1.7; 95% CI = 1.5–2.0) was similar to the SIR in females (1.6; 95% CI = 1.4–1.9). SIRs for biliary tract cancers were 1.4 (95% CI = 1.0–1.9) in males and 1.4 (95% CI = 1.1–1.8) in females. A modest elevation was observed for colon cancer in males (SIR = 1.3; 95% CI = 1.1–1.4). Kidney cancer risk was elevated in both males (SIR = 1.4; 95% CI = 1.2–1.6) and females (SIR = 1.7; 95% CI = 1.4–1.9). Endometrial cancer was also found to occur in excess (SIR = 1.4; 95% CI = 1.2–1.6). Diabetics with reported obesity, who constituted 12% of the cohort, had somewhat higher SIRs for kidney (2.0; 95% CI = 1.5–2.6) and endometrial (2.0; 95% CI = 1.6–2.6) cancers than those without reported obesity (1.4; 95% CI = 1.3–1.6 and 1.2; 95% CI = 1.1–1.4, respectively). The SIR of breast cancer in females was 1.1 (95% CI = 1.1–1.2).

Given the broadly similar risk patterns among males and females, the observed numbers of cancers were pooled, and SIRs were calculated stratifying on the basis of age group at cohort entry (<50 years versus 50 years or more). In view of the age differences for patients with NIDDM versus IDDM entering the cohort from 1987 through 1989, SIRs in the 50 years or more stratum were assumed to reflect cancer risk in a population with predominantly NIDDM, while SIRs in the less than 50 years stratum were assumed to represent cancer risk in a heterogeneous population with a comparatively high percentage of patients with IDDM. To further assess possible differences, SIRs were calculated according to diabetes subtype in the subset entering the cohort from 1987 through 1989.

Primary liver cancer was elevated nearly fivefold in the less than 50 years age-at-entry stratum and threefold in the 50 years or more stratum (Table 2). Pancreatic and kidney cancers were also elevated in both strata, although the 95% CI in the less than 50 years stratum included 1.0 (Table 2). Significant elevations of 40%–50% were observed for biliary tract, endometrial, and vulvar/vaginal (e.g., other female genital) cancers in the 50 years or more stratum, whereas cancers of the mouth and pharynx and of the esophagus were elevated twofold to threefold in the less than 50 years stratum (Table 2).

On the basis of small numbers in the subset entering the cohort from 1987 through 1989, SIRs were suggestive of an elevated risk of liver, biliary tract, and pancreatic cancers in patients with either IDDM or NIDDM. In patients with IDDM, the SIRs were 2.9 (95% CI = 0.6–8.4) for liver, 4.2 (95% CI = 1.1–10.9) for biliary tract, and 3.5 (95% CI = 1.8–6.3) for pancreatic cancers. In patients with NIDDM, the SIRs were 3.1 (95% CI = 2.0–4.7) for liver, 1.8 (95% CI = 0.9–1.3) for biliary tract, and 1.7 (95% CI = 1.2–2.3) for pancreatic cancers. A marginal excess of cancers of the mouth and pharynx (SIR = 1.5; 95% CI = 0.9–2.3) was also observed in patients with NIDDM.

Since hospitalization for diabetes or diabetes-related conditions may have increased the likelihood of detecting prevalent cancers, SIRs were stratified on the number of follow-up years from cohort entry to cancer diagnosis. Liver cancer showed no trend of increasing or decreasing risk with the length of follow-up for either sex alone (Table 3) or both sexes combined. In contrast, pancreatic cancer SIRs decreased from 2.1 (95% CI = 1.9–2.4) for a follow-up time of 1–4 years to 1.3 (95% CI = 1.1–1.6) for a follow-up time of 5–9 years and 1.3 (95% CI = 0.9–1.7) for a follow-up time of 10 years or more (two-sided test for trend; $P<.0001$). The other cancer sites examined showed no significant variation in risk with increasing time interval between cohort entry and cancer diagnosis.

The potentially confounding effects of coexisting medical conditions associated with liver cancer were assessed by stratifying SIRs on the presence or absence of the following diagnoses in hospital records: hepatitis, cirrhosis and other liver disorders (ICD-8 codes 070 and 570–573); alcohol dependence and other alcohol-related conditions (ICD-8 codes 291, 303, 577.1, and 980); cholelithiasis and other disorders of the gallbladder and biliary tract (ICD-8 codes 574–576); jaundice (ICD-8 codes 283 and 785); obesity (ICD-8 code 277); and hemochromatosis (ICD-8 codes 273.2 and 279). Liver cancer SIRs were nearly four times higher in

Table 2. Standardized incidence ratios (SIRs) of cancer in patients hospitalized with diabetes at cohort entry, stratified according to age at entry (Denmark, 1977–1989)

Type of cancer (ICD-7 code[s])*	<50 years			50 years or more		
	No.	SIR	95% CI†	No.	SIR	95% CI
All cancers (140–205)	660	1.1	1.0–1.2	8171	1.1	1.1–1.1
Mouth and pharynx (140–148)	30	1.8	1.2–2.6	142	1.1	0.9–1.3
Digestive organs (150–159)	135	1.7	1.4–2.0	2504	1.3	1.2–1.4
Esophagus (150)	17	3.3	1.9–5.3	76	1.0	0.8–1.3
Stomach (151)	16	1.4	0.8–2.3	303	1.1	1.0–1.3
Small intestine (152)	0	—	—	26	1.4	0.9–2.0
Colon (153)	36	1.3	0.9–1.8	819	1.2	1.1–1.2
Rectum (154)	21	1.2	0.8–1.9	381	1.0	0.9–1.1
Liver (155.0)	17	4.8	2.8–7.7	241	3.2	2.8–3.6
Biliary tract (155.1–.3)	3	1.2	0.2–3.5	117	1.4	1.2–1.7
Pancreas (157)	13	1.4	0.7–2.3	404	1.7	1.5–1.9
Larynx (161)	12	1.6	0.8–2.8	54	0.9	0.6–1.1
Lung (162)	78	1.3	1.0–1.6	885	0.9	0.9–1.0
Breast (170)	87	0.9	0.7–1.1	697	1.2	1.1–1.2
Ovary (175)	15	1.0	0.6–1.6	114	0.8	0.7–1.0
Corpus Uteri (172)	8	0.7	0.3–1.4	223	1.4	1.2–1.6
Cervix (171)	24	1.0	0.7–1.5	68	0.8	0.7–1.1
Other female genital (176)	4	2.5	0.7–6.3	57	1.5	1.1–2.0
Prostate (177)	7	1.0	0.4–2.1	498	0.9	0.8–1.0
Testis (178)	11	0.6	0.3–1.1	12	1.8	1.0–3.2
Kidney (180)	21	1.6	1.0–2.4	301	1.5	1.3–1.7
Bladder (181)	22	0.9	0.6–1.4	471	1.0	0.9–1.1
Melanoma (190)	20	0.7	0.4–1.1	118	1.1	0.9–1.3
Nonmelanoma skin (191)	71	0.8	0.6–1.0	1003	0.9	0.9–1.0
Brain, nervous system (193)	35	1.3	0.9–1.8	124	1.0	0.9–1.2
Thyroid (194)	2	0.5	0.1–1.8	29	1.4	0.9–1.9
Endocrine (195)	1	1.2	0.02–6.9	4	0.8	0.2–2.0
Lymphatic and hematopoietic (200–205)	38	1.0	0.7–1.4	473	1.1	1.0–1.2
Lymphoma (200–202)	16	1.0	0.6–1.6	183	1.1	1.0–1.3
Multiple myeloma (203)	6	1.5	0.5–3.2	93	1.2	0.9–1.4
Leukemia (204)	10	0.8	0.4–1.5	196	1.1	1.0–1.3

*ICD-7 = International Classification of Diseases, seventh revision *(43)*.
†95% CI = 95% confidence interval.

Table 3. Sex-specific standardized incidence ratios (SIRs) of liver cancer, stratified according to years of follow-up, presence of associated diseases, and autopsy diagnosis

Variable	Males			Females		
	No.	SIR	95% CI*	No.	SIR	95% CI
Years of follow-up†						
1–4	90	4.4	3.5–5.4	32	2.3	1.6–3.2
5–9	69	3.6	2.8–4.6	24	1.8	1.2–2.7
⩾10	31	4.2	2.8–5.9	12	2.4	1.2–4.1
Reported presence of associated diseases‡						
No	97	2.6	2.1–3.1	35	1.4	1.0–2.0
Yes	93	9.9	8.0–12.1	34	2.4	1.7–3.4
Incidental diagnosis of liver cancer at autopsy						
Included	190	4.0	3.5–4.6	68	2.1	1.6–2.7
Excluded	132	3.5	3.0–4.2	45	1.6	1.1–2.1

*95% CI = 95% confidence interval.

†Excludes cancers diagnosed less than 1 year after cohort entry.

‡Includes hepatitis, cirrhosis, and other liver disorders, alcohol dependence and other alcohol-related conditions, cholelithiasis and other disorders of the gallbladder and biliary tract, jaundice, obesity, and hemochromatosis.

males and nearly twice as high in females with a co-diagnosis of any of the above conditions compared with SIRs in subjects without any such diagnosis (Table 3), although an elevation in risk was still evident in the latter group.

Autopsy diagnoses of cancer were considered a potential source of detection bias, since diabetics may have higher autopsy rates than the underlying population and, thus, a greater likelihood of incidental cancers reported at death. Therefore, SIRs for primary liver and renal cell cancers, which both have a relatively high frequency of incidental autopsy diagnosis, were re-calculated excluding incidental autopsy-diagnosed cancers from the numerator and from the rates used to generate expected numbers in the denominator. These ratios were compared to SIRs that included autopsy-diagnosed cancers. Sixty-nine percent of male and 66.1% of female cases of primary liver cancer remained in the numerator after exclusion of incidental autopsy-diagnosed cases. The resulting SIRs were slightly lower, although essentially similar to the original ratios (Table 3). A total of 71.4% of renal cell cancers in males and 65.4% in females remained after exclusion of the incidental autopsy-diagnosed cases. However, the SIRs were again very similar. In males, the SIR excluding the autopsy-diagnosed cases was 1.3 (95% CI = 0.9–1.7) compared with a SIR of 1.4 (95% CI = 1.2–1.6) for all renal cell cancers, while in females, the respective SIRs were 1.8 (95% CI = 1.3–2.3) and 1.7 (95% CI = 1.4–1.9).

Diabetes was listed as the sole hospital discharge diagnosis for 25 291 (23.1%) subjects at cohort entry, as the primary but not sole diagnosis for 25 390 (23.2%) subjects, and as a secondary diagnosis for 58 900 (53.7%) subjects. The SIRs of liver and pancreatic cancers were the same in subjects with diabetes as the sole or primary diagnosis and in subjects with diabetes as a secondary diagnosis. SIRs were slightly higher in the secondary diagnosis group for both kidney (1.6; 95% CI = 1.4–1.8 versus 1.4; 95% CI = 1.1–1.6) and endometrial (1.5; 95% CI = 1.3–1.8 versus 1.2; 95% CI = 1.0–1.5) cancers. Circulatory disease constituted 39% of the primary diagnoses in subjects with diabetes as a secondary diagnosis.

A total of 4.8% of subjects were diagnosed with more than one primary cancer during the follow-up period. Unusual clusters of diabetes-associated multiple primaries within subjects were not observed. Among subjects with primary liver cancer, 7.4% had another primary tumor, the most common of which were lung, colorectal, and breast cancers. Among those with kidney cancer, 13% were diagnosed with another primary tumor, one quarter of which were bladder cancers.

Discussion

The main findings in this study indicate that there is an elevated incidence of cancers of the liver, biliary tract, pancreas, kidney, and endometrium in patients hospitalized with a reported diagnosis of diabetes. With the exception of liver cancer, the magnitude of the SIRs for these cancers was small, suggesting that diabetes is unlikely to explain a substantial proportion of them. The elevated incidence of these cancers persisted with increasing years of follow-up, although the SIR of pancreatic cancer declined from 2.1 to 1.3 after 5 years. There were no striking excesses or deficits according to age at cohort entry that would suggest a relationship between the above-named cancers and diabetes subtype, although the modest excess in endometrial cancer was restricted to the 50 years or more stratum. For reasons that are not clear, although chance is possible, elevated risks of oral/pharyngeal and esophageal cancers were observed in cohort members entering prior to the age of 50 years. The preponderance of NIDDM diagnoses (80% of total) among cohort members entering from 1987 through 1989, when diagnostic codes distinguished the two diabetes subtypes, implies that these results mainly reflect cancer risk associated with NIDDM.

This study confirmed the excess of primary liver cancer reported among diabetics in several recent studies from Italy *(4)*, Sweden *(5,7)*, Los Angeles *(6)*, and Japan *(14)* and further demonstrated that the diagnosis of diabetes preceded the diagnosis of liver cancer by many years. While the excess was highest in diabetics with reported medical conditions associated with liver cancer, SIRs were also elevated in the stratum with individuals who lacked these conditions, although the latter SIRs probably underestimate the true risk because national cancer rates applied to the denominator include individuals with these conditions. On the other hand, underreporting of alcoholism, asymptomatic hepatitis infection, and hemochromatosis in hospital records may result in overestimation of risk.

The causal mechanisms for an excess risk of liver cancer in diabetics are unclear, although alcohol consumption may be involved as a risk factor for both conditions. Alcohol consumption has been related to both liver cancer *(15)* and diabetes *(16–19)*, although not all prospective studies *(20–22)* have found an association with NIDDM. Through another mechanism, the liver of diabetics and of obese persons may undergo fatty changes (steatosis), with the potential for necrosis

(steatohepatitis) and fibrotic progression to cirrhosis, perhaps resulting from the cellular accumulation of toxic free fatty acids in insulin-deficient cells *(16,23–25)*.

Although the risk of pancreatic cancer in this study decreased significantly with years of follow-up, a 30% excess remained after 5 or more years. A number of cohort and case–control studies that examined pre-existing diabetes as a risk factor for pancreatic cancer have reported equivocal results *(26)*, and a temporal sequence in which the diagnosis of diabetes precedes the diagnosis of cancer has not been uniformly established *(27,28)*. A direct causal link of diabetes to pancreatic cancer has been questioned because only a small percentage of pancreatic tumors arise in insulin-producing islet cells and most are of exocrine origin. Alternatively, it has been postulated that diabetes and pancreatic cancer are separate, histologically specific responses to a common etiologic factor *(29)*.

Several other cancers were observed to be in excess in this study, including cancers of the kidney in both sexes, of the colon in males, and of the endometrium. In view of the association between diabetes and obesity, it is noteworthy that these cancers were also elevated in a Danish record linkage study of obesity and cancer *(30)*. Obesity and, in particular, central adiposity are recognized risk factors for endometrial cancer *(31)* and postmenopausal breast cancer *(32,33)*, as well as predictors of insulin resistance and hyperinsulinemia *(34)*. Although insulin resistance has been linked to breast cancer risk in one study, independent of body mass index or distribution of adiposity *(35)*, several other studies *(5,36,37)* have not found a relationship of diabetes per se with premenopausal or postmenopausal breast cancer, in concurrence with the results of this study.

Gallbladder cancer, particularly in women, has also been associated with obesity and type of fat distribution, which may reflect a greater prevalence among the obese of carcinogenic risk factors, such as gallstones, cholesterol-supersaturated bile, or high levels of endogenous estrogens *(38)*. In this study, where the SIR of biliary tract cancer was 1.4, diabetes may be functioning as a marker of these risk factors through its association with obesity. A similar effect may explain the excess of colon cancer in male diabetics. In this study, SIRs for gallbladder and colon cancers did not differ among diabetics with and without reported obesity, although underreporting of obesity may have obscured true differences between these strata.

Several studies *(9,39–41)* have reported elevated relative risks of kidney cancer in diabetics, although these risks were not statistically significant or were of borderline significance after adjustment for obesity, a known risk factor for renal cell cancer. An association of renal dialysis with some forms of renal carcinoma and predisposing cysts has been reported *(42)*, and such dialysis may be a relevant risk factor among the subset of diabetics in this cohort who received dialysis for diabetes-related renal disease.

The linkage of national hospital and cancer registries in Denmark to examine cancer outcomes in diabetics has several important advantages. A large sample size was obtained that provided the necessary statistical power to examine site-specific cancer incidence and to analyze further the patterns of risk according to sex and other descriptive variables. The large sample size also provided the opportunity to rule out associations with common cancers such as prostate cancer, which showed no elevation in risk among diabetics. Furthermore, by excluding cancers diagnosed prior to cohort entry from the analyses, a temporal sequence was established in which the diagnosis of diabetes preceded that of cancer. Stratification on the basis of years of follow-up and exclusion of cancers diagnosed within the first year of follow-up or at autopsy demonstrated that detection biases (i.e., increased cancer diagnoses at the time of hospitalization with diabetes or at autopsy) could not fully explain the elevated risks of cancers, such as those of the pancreas, liver, and kidney.

Interpretation of the results of this study is limited by the lack of extensive, reliable data on potentially relevant covariates, including obesity and alcohol consumption, and also by the absence of specific diagnostic codes to distinguish IDDM and NIDDM for most of the time period under observation. Furthermore, because the cohort was established in a hospitalized population, the results may not be generalizable to all diabetics, such as those with asymptomatic or mild disease not requiring hospitalization. Underreporting of diabetes among hospital patients with mild disease also cannot be ruled out. Given the large number of SIRs that were generated, some associations may have appeared due to chance alone. However, chance is unlikely to explain the strong associations that appeared consistently across various subgroups here and in previous studies, such as the excess of primary liver cancer.

This cohort study confirmed the notable excess risk of primary liver cancer in diabetics. The relationship between diabetes and insulin resistance and liver cancer should be explored further in molecular epidemiologic studies where covariates and biologic mechanisms are carefully considered. As the number of years of follow-up increases in which separate ICD codes for IDDM and NIDDM are available in Denmark, future record-linkage cohort studies may prove useful for studying cancer outcomes according to diabetes subtype, bearing in mind that patients who are not dependent on exogenous insulin to sustain life may still be treated with insulin and therefore be assigned the IDDM code.

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Approval for this study was obtained from the Office of Human Subjects Research at the National Institutes of Health and the Institutional Review Board of the Danish Cancer Society.

Manuscript received March 14, 1997; revised June 19, 1997; accepted July 15, 1997.

Molecular Damage in the Bronchial Epithelium of Current and Former Smokers

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Background: **Most lung cancers are attributed to smoking. These cancers have been associated with multiple genetic alterations and with the presence of preneoplastic bronchial lesions. In view of such associations, we evaluated the status of specific chromosomal loci in histologically normal and abnormal bronchial biopsy specimens from current and former smokers and specimens from nonsmokers.** ***Methods:*** **Multiple biopsy specimens were obtained from 18 current smokers, 24 former smokers, and 21 nonsmokers. Polymerase chain reaction-based assays involving 15 polymorphic microsatellite DNA markers were used to examine eight chromosomal regions for genetic changes (loss of heterozygosity [LOH] and microsatellite alterations).** ***Results:*** **LOH and microsatellite alterations were observed in biopsy specimens from both current and former smokers, but no statistically significant differences were observed between the two groups. Among individuals with a history of smoking, 86% demonstrated LOH in one or more biopsy specimens, and 24% showed LOH in all biopsy specimens. About half of the histologically normal specimens from smokers showed LOH, but the frequency of LOH and the severity of histologic change did not correspond until the carcinoma *in situ* stage. A subset of biopsy specimens from smokers that exhibited either normal or preneoplastic histology showed LOH at multiple chromosomal sites, a phenomenon frequently observed in carcinoma *in situ* and invasive cancer. LOH on chromosomes 3p and 9p was more frequent than LOH on chromosomes 5q, 17p (17p13; TP53 gene), and 13q (13q14; retinoblastoma gene). Microsatellite alterations were detected in 64% of the smokers. No genetic alterations were detected in nonsmokers.** ***Conclusions:*** **Genetic changes similar to those found in lung cancers can be detected in the nonmalignant bronchial epithelium of current and former smokers and may persist for many years after smoking cessation. [J Natl Cancer Inst 1997;89: 1366–73]**

Lung cancer is the most frequent cause of cancer deaths in both men and women in the United States *(1)*. Tobacco smoking is accepted as a major cause of cancers of the lung and of several other cancer types *(2)*. As with other epithelial cancers, lung cancer is believed to arise after a series of progressive pathologic changes (preneoplastic lesions) in the bronchial epithelium *(3)*. The sequential preneoplastic changes have been defined for centrally arising squamous cell carcinomas but are poorly documented for small-cell carcinomas and adenocarcinomas. Advanced preneoplastic changes occur far more frequently in smokers than in nonsmokers, and these changes increase in frequency with the amount of smoking, after adjustment for age *(4,5)*. Morphologic recovery occurs after smoking cessation *(4,6)*, although elevated lung cancer risk persists *(7)*. Changes in bronchial epithelium, including metaplasia and dysplasia, have been utilized as surrogate end points for chemoprevention studies *(8,9)*. Risk factors that identify normal or premalignant bronchial tissue at risk for malignant progression need to be better defined.

Many mutations, especially those involving recessive oncogenes, have been described in lung cancers *(10,11)*. Loss of heterozygosity (LOH) analyses utilizing polymorphic microsatellite DNA markers are frequently used to identify allelic losses at specific chromosomal loci. Allelic losses at chromosomal regions 3p, 9p, and 17p occur relatively early during the multistage development of invasive lung cancer *(12–17)*. In addition, there is evidence of more generalized genomic instability in lung cancer and its preneoplastic lesions. Widespread aneuploidy occurs throughout the respiratory epithelium of lung cancer patients *(18)*. Microsatellite alterations are found in many human cancers, including lung cancer, and may serve as clonal markers for early cancer detection *(19,20)*. Microsatellite alterations involve changes in the size of the simple nucleotide repeats of polymorphic microsatellite DNA sequences, resulting in altered electrophoretic mobility of one or both alleles. In lung cancers, such alterations have been reported to occur at frequencies ranging from 0% to 45% *(19,21–23)*. Although the mechanisms underlying microsatellite alterations are currently unknown, they may represent a form of genomic instability *(24)*.

Most previous molecular studies of lung tissue have been performed in material from small numbers of subjects with concurrent lung cancer, and only scant information is available about molecular changes in the respiratory epithelium of smokers without cancer *(15,16,25)*. In this study, we determined the frequency of such alterations at eight chromosomal regions, which are frequently deleted in lung cancers, in the bronchial epithelium of current and former smokers in comparison with nonsmokers.

Materials and Methods

Study Populations

We studied bronchial biopsy specimens obtained by fluorescence bronchoscopy from 63 subjects (21 nonsmokers, 18 current smokers, and 24 former

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See ''Notes'' following ''References.''

smokers) (Table 1). All subjects were recruited at the British Columbia Cancer Agency, Vancouver, Canada, as part of an Institutional Review Board-approved clinical trial to study the effect of smoking on the respiratory epithelium. All participants gave written informed consent before enrollment into the study. Because of our inability to enroll older nonsmokers, the control subjects consisted of 16 relatively young, healthy volunteers, three subjects who had bronchoscopy for chronic cough, and two individuals with a history of occupational exposure to asbestos. Subjects were categorized as to smoking status as follows: (*a*) Nonsmokers were subjects who had smoked fewer than 365 cigarettes in their lifetime, (*b*) current smokers were subjects who had smoked more than 365 cigarettes in their lifetime and who either were currently smoking or had stopped smoking within the previous 12 months, (*c*) former smokers were subjects who had smoked more than 365 cigarettes in their lifetime and who had stopped smoking for longer than the previous 12 months, and (*d*) lifetime smokers (also referred to as ''smokers'') consisted of a combination of current and former smokers. Because almost all adults have at least a minimal exposure to cigarette smoking, we arbitrarily chose a 365-cigarette exposure, equivalent to one cigarette per day for a year, as the distinction between smokers and nonsmokers. All smokers had smoked more than 20 pack-years (pack-years = number of packs per day multiplied by the number of years of smoking), except for three subjects (1, 10, and 12 pack-years). Most former smokers (72%) had ceased smoking for 5 years or longer (mean, 11 years; range, 1–48 years). Sixteen smokers had a history of prior carcinoma *in situ* (CIS) or invasive lung cancer.

Of the subjects, 49 (78%) were males and 14 (22%) were females. The mean age of the smokers was 63 years (range, 26–88 years). Because we were unable to recruit older nonsmokers, the average age of the nonsmokers was considerably lower (mean, 34 years; range, 21–60 years). Other relevant subject information is presented in Table 1.

Fluorescence Bronchoscopy

Fluorescence bronchoscopy was performed by one of us (S. Lam), as previously described *(26,27)*. The procedure was performed on the subjects under local anesthesia with the use of the LIFE-Lung device following conventional white-light examination. Briefly, upon illumination of the bronchial surface with a blue light (405–442 nm), the autofluorescence intensity progressively decreases as the tissue changes from normal to metaplasia, dysplasia, CIS, and invasive cancer. Lesions as small as 1 mm in surface diameter were localized, and biopsy specimens were taken under direct vision. Specimens were taken from all areas of the bronchial tree that were suspicious for moderate dysplasia or worse. In addition, at least two biopsy specimens from widely separated areas of normal or minimally abnormal fluorescence were taken.

Identification of Preinvasive Lesions

Sections (5 μm thick) of bronchial biopsy tissues were stained with hematoxylin–eosin and examined by two reference pathologists (A. F. Gazdar and J. LeRiche) and scored with the use of published criteria for the histologic identification of epithelial premalignant lesions *(3)*. In case of disagreement, a consensus diagnosis was achieved with help of the third pathologist (I. I. Wistuba). Pathologic diagnoses were categorized as follows: category 1, normal bronchial epithelium; category 2, hyperplasia (goblet cell or basal cell type) or simple squamous metaplasia without dysplasia; category 3, mild dysplasia; category 4, moderate or severe dysplasia; and category 5, CIS.

Microdissection and DNA Extraction

Microdissection and DNA extraction were performed from specimens mounted on microscope slides, as previously described *(13)*. Precisely identified areas of normal and abnormal bronchial epithelia were microdissected under microscopic visualization. Microdissected stromal cells from the same slides provided constitutional DNA. Biopsy specimens containing a total of at least 300–800 epithelial cells in one or more sections were regarded as adequate for analysis and were microdissected. After DNA extraction, 5 μL of the digested samples, containing DNA from at least 50 cells, was used for each polymerase chain reaction (PCR) reaction.

Polymorphic DNA Markers and PCR–LOH Analysis

To evaluate LOH and microsatellite alterations, we used primers flanking dinucleotide and multinucleotide microsatellite repeat polymorphisms located at the following genes or chromosomal locations: 3p14.2 (FHIT [fragile histidine triad] locus, D3S4103), 3p14.3–21.1 (D3S1766), 3p21 (D3S1447, D3S1478, and D3S1029), 3p22–24.2 (D3S2432, D3S1351, and D3S1537), 5q22 (L5.71CA), 9p21 (D9S171 and IFNA [interferon alfa]), 13q14 (RB [retinoblastoma] gene, dinucleotide repeat at intron 2, and tetranucleotide repeat at intron 20), and 17q31.1 (TP53 [p53] gene, the dinucleotide repeat TP53, and a pentanucleotide repeat). With four exceptions *(28–30)*, most of the primer sequences were obtained from the Human Genome Database. Allelic loss was determined by modifying a previously described *(13)* PCR-based assay, generating $^{32}PO_4$-labeled amplification products, as follows: (*a*) Nested PCR methods were used; (*b*) for detection of loci within the TP53 and FHIT genes and the 3p21 region, hot-start PCR (TaqStart Antibody; CLONTECH Laboratories, Inc., Palo Alto, CA) was used. In individual subjects, only informative markers that demonstrated constitutional heterozygosity were tested for LOH. There were no statistically significant differences in heterozygosity rates according to chromosomal regions for any subject category. LOH was scored by visual detection of complete absence of one allele. Microsatellite alterations were detected by a shift in the mobility of one or both alleles (Fig. 1). After generation of the initial data and breaking of the histologic code (*see below* for information on binding), we confirmed that LOH and microsatellite alterations were not artifacts by re-examining all changes in histologically normal biopsy specimens by repeat PCR analysis, frequently from an independent microdissection.

Calculation of Indices for Documenting Extent of Molecular Changes

Because heterozygosity at the different loci varied between subjects, the number of chromosomal regions tested in subjects varied. Thus, indices were created to compare molecular changes between subjects and between biopsy specimens. The fractional regional loss index for individual biopsy specimens (FRL-biopsy) and the fractional regional loss index for all biopsy specimens from an individual subject (FRL-subject) were calculated as follows:

$$\text{FRL-biopsy index} = \frac{\text{total number of chromosomal regions with LOH}}{\text{total number of informative}}, \text{ for any one biopsy.}$$

Table 1. Characteristics of patients and control subjects: smoking histories, clinical information, and biopsy specimens

Subject categories	No.	Pack-years,* mean (range)	Years of smoking cessation, mean (range)	Sex, M:F†	Age, y		Biopsy specimens studied	
					Mean (range)	Median	Total	Mean (range)
Lifetime nonsmokers	21	NA‡	NA‡	15:6	34 (21–60)	29	67	3.1 (1–7)
Lifetime smokers	42§	49 (1–132)	—	34:8	63 (26–88)	61	188	4.5 (2–9)
Current smokers	18	44 (12–77)	0	14:4	60 (34–83)	59	92	5.1 (2–9)
Former smokers	24	54 (1–132)	11 (1–48)	20:4	65 (26–88)	63	96	4.0 (2–9)
All subjects	63	—	—	49:14	53 (26–88)	58	255	4.1 (1–9)

*Pack-years = number of packs of cigarettes smoked per day multiplied by the number of years of smoking.
†M = male; F = female.
‡Not applicable.
§Two current smokers and 14 smokers had a previous lung cancer.

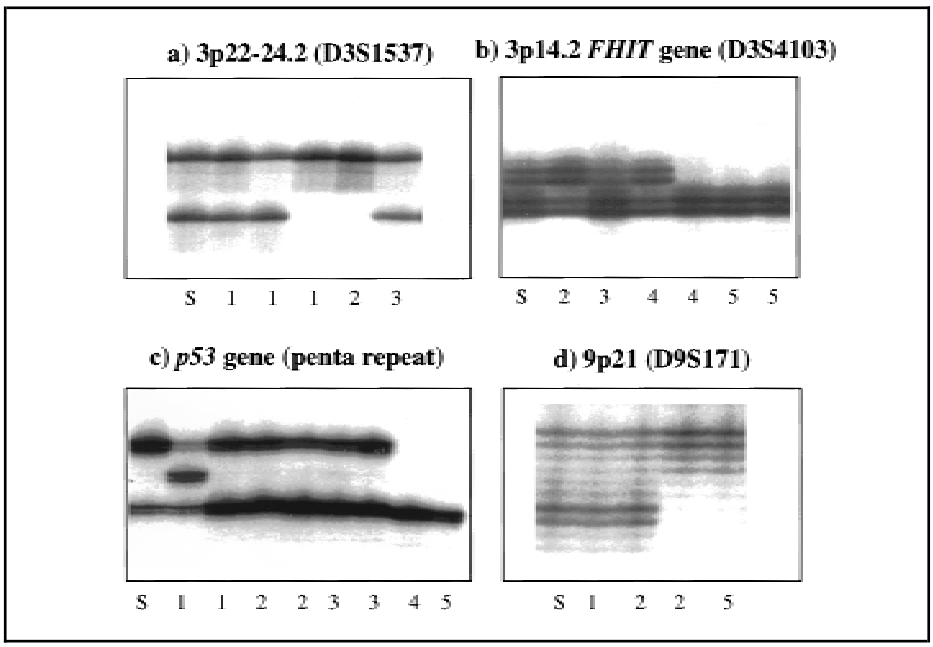

Fig. 1. Representative autoradiographs of microsatellite DNA analyses involving biopsy specimens from four smoker subjects **(a–d),** showing loss of heterozygosity at chromosomal regions 3p22–24.2 **(a),** 3p14.2 (FHIT gene) **(b),** 17p (TP53 gene) **(c),** and 9p21 **(d).** S = normal stromal cells; 1 = histologic category 1 (normal bronchial epithelium); 2 = histologic category 2 (hyperplasia or simple squamous metaplasia without dysplasia); 3 = histologic category 3 (mild dysplasia); 4 = histologic category 4 (moderate or severe dysplasia); 5 = histologic category 5 (carcinoma *in situ*). In panel **c,** in the TP53 pentanucleotide repeat marker, a microsatellite alteration is evident in normal epithelium (category 1, lane 2). *See* text for more details.

$$\text{FRL-subject index} = \frac{\text{total number of chromosomal regions with LOH summed for all}}{\text{total number of informative summed for all}}, \text{ for any one subject.}$$

Statistical Analyses

Pathologists and laboratory staff were blinded as to subject category and other clinical information until the data were merged for analysis. Data were analyzed by use of chi-squared methods for proportions *(31).* Because of the distribution of the biopsy and patient indices, the nonparametric Wilcoxon test *(31)* was used to compare the groups. Spearman correlation coefficients were calculated between the number of regions with LOH and age and pack-years of smoking *(31).* The cumulative binomial test *(32)* was used to examine the likelihood that a particular event (loss of the same allele in paired biopsy specimens) occurs at a particular probability when observed in repeated trials. When the results are compared with a chance occurrence or nonoccurrence, the particular probability of comparison is .5. All reported *P* values are two-sided.

Results

Histologic Changes

A total of 315 biopsy specimens were obtained from 63 subjects (average, five per subject; range, one to nine per subject). Two hundred fifty-five specimens (81%) contained adequate numbers of surface epithelial cells to perform multiple DNA analyses (Table 1). Ninety-two biopsy specimens were from current smokers (mean of 5.1 per subject), 96 were from former smokers (mean of 4.0), and 67 were from nonsmokers (mean of 3.1). While the nonsmokers were significantly younger than the smokers, sample adequacy was similar in smokers and nonsmokers (Table 1).

The distribution of histologic categories of biopsy specimens was significantly different ($P<.001$) between smokers and nonsmokers (Fig. 2, A). In nonsmokers, 65 (97%) of 67 biopsy specimens demonstrated normal or slightly abnormal changes (categories 1 and 2), two (3%) biopsy specimens demonstrated mild dysplasia (category 3), and none demonstrated more severe changes (categories 4 or 5). In contrast, 96 (51%) of 188 biopsy specimens from 33 (79%) of 42 smoker subjects demonstrated histologic categories 3–5, and only 26 (14%) of 188 biopsy specimens were normal. In addition, only four (4%) of 92 biopsy specimens from current smokers were normal compared with 24 (25%) of 96 biopsy specimens from former smokers ($P<.001$).

Comparison of Molecular Changes Between Nonsmokers and Smokers

A most striking finding was the complete absence of molecular changes in every biopsy specimen from every nonsmoker subject. In contrast, a very high frequency of LOH at one or more chromosomal regions was detected in 36 (86%) of 42 smokers and in 91 (48%) of 188 of their biopsy specimens ($P = .0001$) (Figs. 2 and 3). There was a modest correlation between the number of molecular changes per subject (FRL-subject index) and smoking exposure ($r = .34$). Of interest, the difference in the mean FRL-subject index between current (0.19) and former smokers (0.18) was not significant ($P = .98$) (Fig. 2, B). In addition, the difference in the mean FRL-subject index between subjects with a history of prior lung cancer (0.20) and those without such a history (0.18) was not significantly different ($P = .12$).

Of interest, 10 subjects (five current smokers and five former smokers; 24% of the subjects who smoked) demonstrated LOH at one or more chromosomal regions in all of their biopsy specimens. In contrast, in six subjects (one current smoker and five former smokers), no LOH was detected in any biopsy specimen (Fig. 2, B).

Because the smokers tended to be older and, in fact, there were no nonsmokers above 60 years of age, we attempted to use various multivariate models to control for the effect of age so that the effect of smoking could be estimated without confounding by the age differences. Because of the differences in the age distributions, these models were not fully successful for this purpose. However, none of the three nonsmokers over 45 years of age had any mutations. In contrast, among four smokers under 45 years of age, all had multiple mutations in multiple biopsy specimens.

Correlation Between Molecular Alterations and Histologic Changes in Smokers

We correlated the fraction of regional loss per biopsy specimen with the histologic category in smokers. The data, sum-

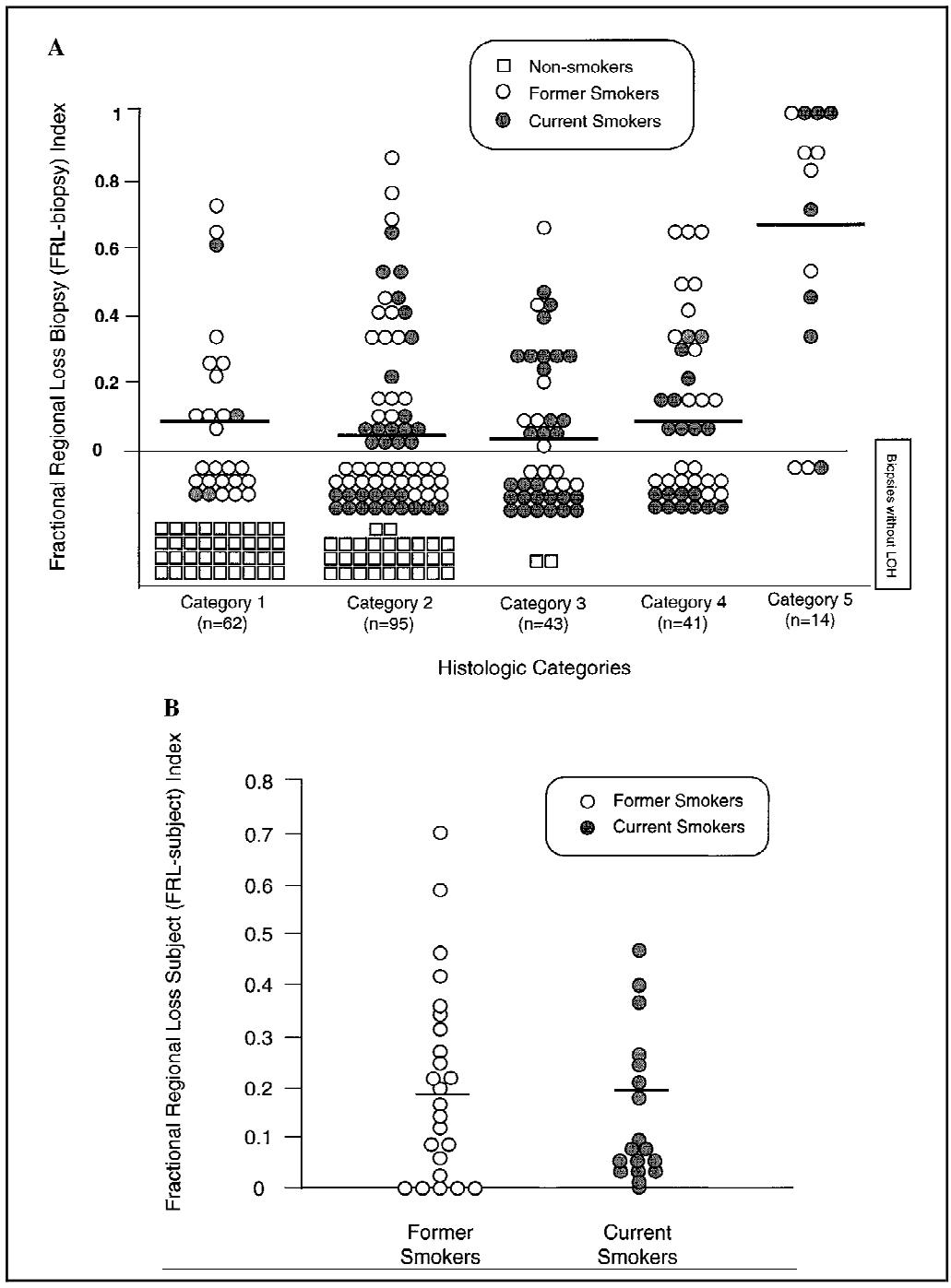

Fig. 2. A) Loss of heterozygosity (LOH) in individual biopsy specimens according to smoking status and histologic categories. LOH is expressed in terms of the fractional regional loss biopsy (FRL-biopsy) index (i.e., the fraction of chromosome regions showing LOH in each biopsy specimen) (range, 0–1). Horizontal bars indicate the mean for each histologic category. **B**) The FRL-subject (i.e., fractional regional loss for all biopsy specimens from an individual subject) index distribution in current and former smoker subjects. Horizontal bars represent the mean for each group of subjects. *See* text for more details.

marized in Fig. 2, A, demonstrate that the mean FRL-biopsy indices were similar (0.13–0.15) from category 1 (normal epithelium) to category 4 (moderate or severe dysplasia) until a significant rise (0.61) occurred in category 5 (CIS) ($P = .001$).

Another important observation was the presence of frequent LOH in histologically normal biopsy specimens (category 1; Fig. 2, A). LOH at one or more chromosomal regions was detected in 13 (50%) of 26 histologically normal biopsy specimens taken from 10 (53%) of 19 smokers.

LOH was detected more frequently at certain chromosomal sites than at others (Fig. 3, A and B). The most frequent allelic losses occurred at one or more chromosome 3p regions (38% of all biopsy specimens) and at chromosome 9p21 (23% of all biopsy specimens). The least frequent change was LOH at chromosome 5q (2% of all biopsy specimens, none of which were in normal epithelium). LOH at the TP53 (12% of biopsy specimens) and RB (18%) genes occurred at intermediate frequencies.

We tested whether there were differences between the four specific chromosome 3p regions (3p14.2 [FHIT gene], 3p14.3, 3p21, and 3p22–24.2) suspected to harbor tumor suppressor genes. Apart from 3p14.2 (FHIT gene), LOH at the other chromosome 3p regions was detected in histologically normal epithelium and was quantitatively similar until the CIS stage, when a statistically significant increase in frequency was noted for all regions (Fig. 3, B). In contrast, LOH at 3p14.2 (FHIT gene) was first detected at the later stage of mild dysplasia.

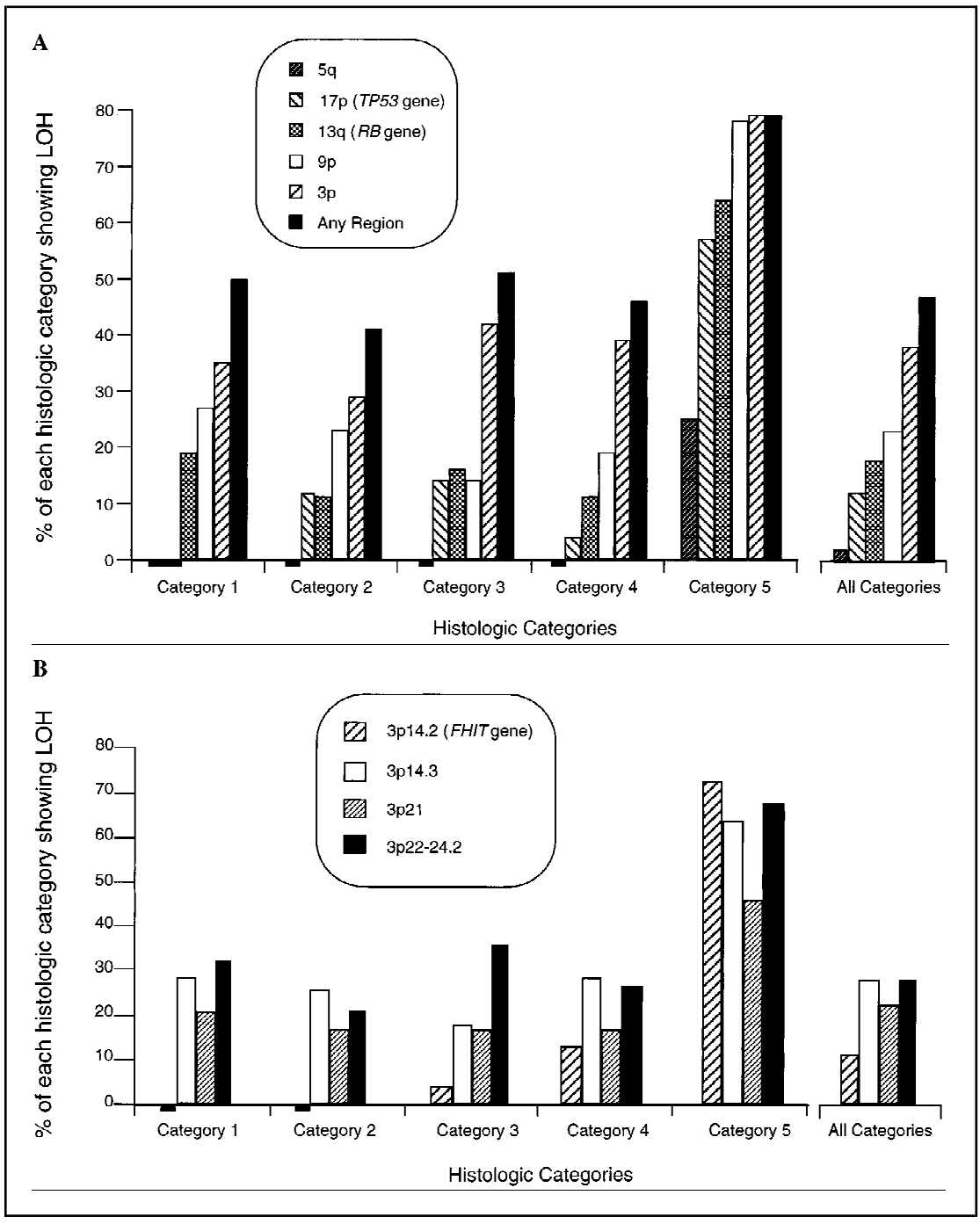

Fig. 3. A) Relationship between loss of heterozygosity (LOH) at individual chromosomal regions according to histologic categories in smokers. **B**) LOH at specific regions of chromosome 3p according to histologic categories in smokers. *See* legend to Fig. 1 for histologic category definitions.

Allele-Specific Loss

We have previously described the phenomenon that we labeled allele-specific loss (ASL) indicating that the identical allele is lost in widely separated areas of the respiratory epithelium *(13,14)*. In this study, we found the same phenomenon when two or more biopsy specimens from the same subject demonstrated losses of the same marker(s). In such comparisons of paired biopsy specimens, ASL was noted in 118 (93%) of 127 cases. According to the cumulative binomial test, the probability of this finding happening by chance is 1×10^{-25}.

Microsatellite Alterations in Smokers

A high frequency of microsatellite alterations at one or more chromosomal loci was detected only in smokers (27 [64%] of 42 subjects and 46 [24%] of 188 biopsy specimens). Of interest, microsatellite alterations at one or more chromosomal loci were detected in four (15%) of 26 histologically normal biopsy specimens occurring in four (21%) of 19 smokers. The frequencies of microsatellite alterations did not alter with increasing histopathologic changes. There were no statistically significant differences in the frequencies of microsatellite alterations between current and former smokers or between subjects with or without a history of prior carcinoma.

Discussion

Because most lung cancers are attributable to smoking, we investigated molecular changes in the normal and abnormal bronchial epithelia of smokers and nonsmokers. Fluorescence bronchoscopy

was used to identify and to obtain biopsy specimens from multiple areas of histologically normal and abnormal bronchial epithelia in nonsmokers, current smokers, and former smokers. After careful microdissection of the epithelium, extracted DNA was analyzed by PCR for LOH at eight chromosomal regions frequently deleted in lung cancers. These analyses utilized 15 polymorphic microsatellite markers. In nonsmokers, almost all (97%) of the biopsy specimens demonstrated normal or slightly abnormal histology. In contrast, biopsy specimens from both current and former smokers demonstrated the entire spectrum of preneoplastic pathologic changes associated with lung cancer. A significantly higher percentage of biopsy specimens from former smokers (25%) than from current smokers (4%) had normal histology. These morphologic findings are consistent with previous observations that dysplasia and CIS occur less frequently in nonsmokers than in smokers *(4)* and that histologic recovery of the bronchial epithelium may occur relatively rapidly after smoking cessation *(4,6)*.

While the 21 nonsmokers consisted of healthy volunteers as well as subjects being investigated because of chronic cough or occupational exposure to asbestos, no molecular changes (either LOH or microsatellite alterations) were present in any of the 67 biopsy specimens analyzed. In contrast, extensive LOH, frequently of multiple regions, was present in nearly half (48%) of the biopsy specimens from smokers, including 50% of histologically normal biopsy specimens. Most smokers (86%) had allelic loss in at least one biopsy specimen, and 10 subjects (24%) demonstrated allelic loss in every biopsy specimen analyzed. In addition, microsatellite alterations were present in at least one biopsy specimen from 64% of the smokers.

Interpretation of the findings on smoking is potentially limited by the differing age distribution of the smokers and nonsmokers included in the study. The smokers tended to be older; in fact, there were no nonsmokers above 60 years of age. We attempted to use various multivariate models to control for the effect of age so that the effect of smoking could be estimated without confounding by the age differences. Because of the differences in the age distributions, these models were not fully successful for this purpose. However, the complete absence of genetic changes in the nonsmokers across the age span from 20 to 60 years indicates that the substantial genetic changes found in the smokers cannot be attributed to age alone. Even in the older nonsmokers (three of whom were above 45 years of age), there was no indication of genetic change.

As a result of public health campaigns in the United States, there has been a substantial reduction in the percentage of adults who smoke. From published figures, it can be estimated that there are approximately equal numbers of smokers and former smokers nationwide (about 43 million in each category) *(33)*. At the University of Texas M. D. Anderson Cancer Center, Houston, more than half of the recently diagnosed lung cancers arise in former smokers, and nearly 50% of these had quit smoking more than 5 years previously *(34)*. From the current smoking trends, it appears that former smokers will account for a growing percentage of all patients with lung cancer. Multiple mutations are found in invasive lung tumors, and, presumably, the diminished risk of former smokers is due to a decrease in the accumulation of new mutations in the bronchial epithelium. Somewhat surprisingly, we found no statistically significant differences in the frequencies or patterns of allelic loss between current and former smokers, and multiple molecular abnormalities were found in biopsy specimens from subjects who had quit 10–48 years previously. These findings suggest that molecular changes, unlike histologic changes, may persist long after smoking cessation. Our findings are consistent with the maintained increased risk of lung cancer in former smokers *(7)*.

Subjects with previous aerodigestive cancers (including those of the lung) are at greatly increased risk for the development of second cancers *(35–38)*. Thus, another unexpected finding was the lack of significant differences in molecular changes between subjects with and without a previous history of lung cancer.

The development of epithelial cancers requires multiple mutations *(39)*, the stepwise accumulation of which may indicate a mutator phenotype *(40,41)*. Thus, it is possible that those preneoplastic lesions that have accumulated multiple mutations are at highest risk for progression to invasive cancer. We found that 12% of histologically normal biopsy specimens had allelic loss equal to or greater than that present in CIS lesions. These findings suggest that CIS and invasive tumors may arise directly either from normal epithelium or from abnormal epithelium, without passing through the entire histologic sequence (parallel theory of cancer development) *(42)*. In contrast, three CIS lesions lacked allelic loss in any of the regions studied. While no published data exist for CIS of the respiratory tract, multiple studies of the natural history of uterine cervical CIS indicate that only a subset progresses to invasive cancer [reviewed in *(43)*]. Our findings suggest that CIS and other histologically normal or abnormal foci having multiple regions of allelic loss are at increased risk of progressing to invasive cancer.

In colon cancer, the accumulation of mutations during the preneoplastic process is not random but usually follows a pattern *(44)*. Similarly, in lung cancer, the developmental sequence is not random, with LOH at one or more chromosome 3p regions and at chromosome 9p21 being early events and RAS mutations occurring relatively late *(12–15)*. Our finding that losses at chromosomes 3p and 9p are frequent and early events in current and former smokers without lung cancer are consistent with these observations. In contrast, LOH at chromosome 5q, a relatively frequent event in invasive lung cancer *(45,46)*, was detected only in the CIS stage. The short arm of chromosome 3 (3p) contains several discrete regions, including 3p12, 3p14, 3p21, and 3p24–25, which are deleted in lung and other cancers and which are each believed to harbor recessive oncogenes *(11)*. Deletions at one or more of these sites were frequently detected in histologically normal or slightly abnormal bronchial biopsy specimens.

We have previously demonstrated in patients with lung cancer that widely separated regions of the bronchial mucosa may demonstrate loss of the same allele of a polymorphic marker *(13,14)*, a phenomenon that we have referred to as allele-specific loss (ASL). In this study, ASL was noted in 93% of paired comparisons. According to the cumulative binomial test, the probability of this finding happening by chance is 1×10^{-25}. While ASL may represent clonality, we have

suggested that alternate explanations may exist *(13)*.

Alterations in microsatellite size are another genetic change associated with many cancers, including lung cancers *(19,21–23)*. The relationship between microsatellite alterations and DNA repair mechanisms in lung cancer has not been established, but these alterations probably represent evidence of some form of genomic instability *(24)*. Nevertheless, microsatellite alterations are attractive candidates for the early molecular detection of cancer *(19,20)*. We found microsatellite alterations in the normal and abnormal epithelia of smokers but not in nonsmokers. Unlike LOH, the frequency of microsatellite alterations did not increase with more advanced histologic categories.

While this study was under review, a similar study was reported *(25)*, describing frequent deletions in bronchial biopsy specimens from current and former smokers. Our results, which are in agreement with these findings, indicate that multiple clonal outgrowths of molecularly altered cells are widely distributed in the bronchial epithelium of smokers and that they persist for many years after smoking cessation. Our findings suggest the hypothesis that identifying biopsy specimens with extensive or certain patterns of allelic loss may provide new methods for assessing the risk of developing invasive lung cancer in smokers and for monitoring their response to chemoprevention. As with all diagnostic tests, these concepts will need to be validated in clinical trials.

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Notes

Supported by Public Health Service contract N01CN45580-01 and grant 1P50CA70907–01 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

We thank Jing Xu for assistance with the statistical analysis.

Manuscript received April 21, 1997; revised July 15, 1997; accepted July 16, 1997.

Geographic Variation in Breast Cancer Incidence Rates in a Cohort of U.S. Women

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Background: **Breast cancer mortality and incidence rates vary by geographic region in the United States. Previous analytic studies have measured mortality, not incidence, and have used regional prevalences to control for geographic variation in risk factors rather than adjusting for risk factors measured at the level of the individual. We prospectively evaluated regional variation in breast cancer incidence rates in the Nurses' Health Study and assessed the influence of breast cancer risk factors measured at the individual level.** ***Methods:*** **The Nurses' Health Study cohort was established in 1976 when 121 700 female nurses aged 30–55 years living in 11 U.S. states were enrolled. These states represent all four regions of the continental United States. We identified 3603 incident cases of invasive breast cancer through 1992 (1 794 565 person-years of follow-up). We calculated relative risks (RRs) adjusted for age and for age and established risk factors (i.e., multivariate-adjusted analysis), comparing California, the Northeast, and the Midwest with the South.** ***Results:*** **For premenopausal women, there was little evidence of regional variation in breast cancer incidence rates, either in age-adjusted or in multivariate-adjusted analyses. For postmenopausal women in California, age-adjusted risk was modestly elevated (RR = 1.24; 95% confidence interval [CI] = 1.05–1.47); after adjusting for age and for established risk factors, the excess rate in California was attenuated by 25% (RR = 1.18; 95% CI = 1.00–1.40). No excess of breast cancer incidence was observed for postmenopausal women in either the Northeast or the Midwest.** ***Conclusions:*** **Little regional variation in age-adjusted breast cancer incidence rates was observed, with the exception of a modest excess for postmenopausal women in California. Adjustment for differences in the distribution of established risk factors explained some of the excess risk in California. [J Natl Cancer Inst 1997; 89:1373–8]**

Mortality rates from breast cancer vary by geographic region within the United States. Average annual age-adjusted mortality rates ranged from 18.0 per 100 000 women in Hawaii to 35.7 per 100 000 women in the District of Columbia during the period 1987 through 1991 *(1)*. In regional analyses, mortality rates among women older than 50 in the northeastern region have been reported to be from 20% to 50% higher than those in the southern United States *(2–4)*. This pattern, along with the rise in incidence rates

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See ''Notes'' following ''References.''

throughout most of this century *(5)*, evidence that higher rates are found in urban areas *(2,6)*, and reports of suspected cancer clusters *(7–9)* have led to speculation about environmental pollutants as causes of breast cancer. However, there is also evidence that established risk factors for breast cancer, such as fertility rates *(10)*, delayed childbearing *(2,4)*, economic status and educational level *(11)*, exogenous hormone use *(4)*, and alcohol consumption *(12)*, vary modestly between regions. Furthermore, regional differences in mortality could be due to differences in the prevalence of early detection [e.g., mammographic screening practices vary modestly by region *(13)*] and/or treatment of incident breast cancers *(14–16)*. In most previous nationwide studies, mortality rates have been used, and geographic variation of potentially confounding factors was controlled for by using regional prevalences of these factors, rather than adjusting for individually measured risk factors.

We evaluated prospectively the regional variation of invasive breast cancer incidence in the Nurses' Health Study controlling for breast cancer risk factors collected at the individual level. The Nurses' Health Study represents a single occupational group; potential confounders related to socioeconomic status that are notoriously difficult to adjust for directly are at least partially removed by restricting to this narrower socioeconomic stratum.

Methods

Study Population

The Nurses' Health Study is an ongoing prospective cohort study established in 1976 when 121 700 registered nurses completed a mailed questionnaire that included items about risk factors for breast cancer and other diseases. At enrollment, the participants were between the ages of 30 and 55 years old and resided in 11 large states (California, Connecticut, Florida, Maryland, Massachusetts, Michigan, New Jersey, New York, Ohio, Pennsylvania, and Texas). These states were originally chosen based on their size and the approval of the study by the state nursing associations. No restrictions were made on the basis of ethnicity or race; however, the participants were primarily Caucasian (approximately 97%), reflecting the ethnic background of women trained as registered nurses. Every 2 years, participants completed follow-up questionnaires to update information on risk factors for breast cancer and to report the occurrence of breast cancer and other illnesses.

The subjects included in this analysis were the 118 349 women who did not report breast cancer or other cancers (with the exception of nonmelanoma skin cancer) at baseline in 1976.

Assessment of Exposure

Place of residence was defined as the region of the country in which the participant lived in 1976, the beginning of the study. We grouped the 11 states represented by this cohort into four regions based on census definitions *(17)*—Northeast (Connecticut, Massachusetts, New York, New Jersey, and Pennsylvania), Midwest (Michigan and Ohio), South (Florida, Maryland, and Texas), and West (California). In 1976, 14 674 (12.4%) of the cohort lived in California, 68 921 (58.2%) lived in the Northeast, 21 702 (18.3%) lived in the Midwest, and 13 052 (11.0%) lived in the South. The nurses lived throughout the 11 states; the county-specific population distribution of the cohort reflected that of the general population of white women of the same age range, with the exception of some underascertainment of women in large urban counties in the Northeast and in small counties in the South. To account for duration of exposure, we also defined residence by region in which the participant lived in 1986, 10 years after the start of the study. In some analyses, we restricted the cohort to the 110 741 participants (94%) who lived in the same region in 1976 and in 1986, thereby defining a stable population. In further analyses, we restricted to the 62 672 women (53%) who reported in 1992 that they had lived in the same region at birth, at age 15 years, and at age 30 years.

Assessment of Outcome

Diagnoses of breast cancer were reported on the biennial follow-up questionnaires. We attempted to contact nonrespondents by telephone and identified deaths through next of kin or searches of the National Death Index. For each case of breast cancer reported, we requested permission to obtain medical records and pathology reports to confirm the diagnosis. Because the accuracy of self-reported breast cancer was extremely high *(18)*, we included in this report the small number of cases for whom pathology reports were not obtained (n = 191). In the majority of analyses, we considered incident cases of invasive breast cancer only. In one analysis, we included incident cases of *in situ* carcinoma of the breast, and we also analyzed breast cancer mortality, as determined by review of death certificates and medical records.

Assessment of Breast Cancer Risk Factors

We obtained information on known and suspected risk factors for breast cancer in 1976 and updated the information at the beginning of each 2-year period, as appropriate. We included the following risk factors in the multivariate models: age, menopausal status, age at menopause, age at menarche, parity, age at first full-term pregnancy, use of oral contraceptives, use and duration of use of postmenopausal hormone therapy, history of breast cancer in a mother or a sister, history of benign breast disease, height, current body mass index (weight [kg]/height$[m]^2$), and body mass index at age 18 years. We classified a woman as postmenopausal from the time she returned a questionnaire on which she reported natural menopause or hysterectomy with bilateral oophorectomy. Women reporting hysterectomy without bilateral oophorectomy were assumed to be postmenopausal at the age when natural menopause had occurred in 90% of the cohort (54 years for current cigarette smokers and 56 years for nonsmokers); otherwise, we considered them to be of uncertain menopausal status.

We collected information on alcohol consumption prospectively beginning in 1980 *(19)*, and it was updated in 1984, 1986, and 1990. In 1988 we inquired as to whether the participant had ever had a mammographic examination.

Allocation of Person-Time

Follow-up began on June 1, 1976. Each participant contributed person-time to the analysis up until June 1, 1992, until date of diagnosis of breast cancer (date of death from breast cancer for the mortality analysis), or until the date of death from other causes, whichever came earlier. In all analyses, except those involving mortality, women who reported a diagnosis of cancer other than nonmelanoma skin cancer on any questionnaire were excluded from subsequent follow-up at the beginning of the next follow-up cycle. For analyses adjusting for alcohol intake, we began follow-up in 1980 (when alcohol consumption was first assessed) and limited the cohort to the women who were cancer-free at the start of the 1980 follow-up and who provided detailed dietary information in 1980 (n = 89 512). Person-time for each participant was allocated to their region of residence in 1976. Each individual's risk factor status was updated at the beginning of each 2-year period on the basis of information provided on the follow-up questionnaires. The follow-up rate was similar between regions and averaged 95% of potential person-time.

We performed all analyses within the entire cohort and separately among premenopausal and postmenopausal women. In 1976, 22 990 women reported that they were postmenopausal and entered the postmenopausal follow-up in the period 1976 through 1978. As women became postmenopausal during follow-up, their person-time was added to the postmenopausal analysis. By the start of the 1990 through 1992 time period, 71 070 women were defined as postmenopausal. Women who started follow-up as premenopausal (84 692 in 1976) were excluded from the premenopausal analysis as their menopausal status changed. Women with missing or uncertain menopausal status during a given time period were excluded from the stratified analysis during that time period.

Regional Distribution of Risk Factors

To assess the regional distribution of breast cancer risk factors and their potential to confound the region/breast cancer relationship, we calculated the proportion of person-time in each covariate category by menopausal status, standardized to the age distribution of the premenopausal or postmenopausal cohort. For the risk factors assessed for the full period of follow-up, we used the age distribution of the entire postmenopausal cohort and of the entire premenopausal cohort to standardize the postmenopausal and premenopausal prevalences, respectively. For alcohol use, we used the age distributions of the 1980 cohort with dietary data. History of mammography was first asked in 1988. Therefore, we calcu-

lated the percent of women who answered the 1988 questionnaire and reported ever having had a mammographic examination.

Incidence Rates and Comparison to National Rates

To calculate the age-standardized incidence rates, we divided the number of incident breast cancers by the person-time of follow-up and standardized the regional incidence rates to the age distribution of the entire cohort at baseline. To assess the comparability of the Nurses' Health Study breast cancer incidence with the national incidence rates of invasive breast cancer, we calculated the expected number of cases using age-specific incidence rates observed by the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER)[1] Program over the period 1976 through 1990 *(20)*, standardized to the age distribution of the Nurses' Health Study. The SEER program consists of data from nine population registries for cancer incidence in various locations that represent approximately 10% of the U.S. population.

Multivariate Analyses

We used the likelihood ratio test, comparing the model with indicator variables for both age and region with the model with only age, to evaluate the contribution of region to the model and address the general question of whether regional variation existed in this cohort. To be consistent with previously published research from the NCI, we chose the women residing in the South as the reference group when comparing incidence between regions *(4)*. We calculated relative risks (RRs), dividing the incidence rate in each region by the incidence rate in the South. To control simultaneously for potential confounding factors we conducted proportional hazard analyses *(21)* by using a pooled logistic regression model *(22,23)* with indicator variables for each region for each category of each breast cancer risk factor and for 2-year intervals of calendar time. We calculated the 95% confidence intervals (CIs) for each RR.

To test whether including the small numbers of non-Caucasians in the analysis altered our conclusions, we compared our overall results with results obtained by restricting the cohort to white women who answered a question on ethnicity (n = 95 672) in 1992.

Results

Distribution of Risk Factors in the Cohort

The age-standardized prevalences of established and potential risk factors for breast cancer varied modestly across region for both premenopausal and postmenopausal women. (These differences were statistically significant; however, even very small differences are statistically significant with such a large sample size.) Prevalences for postmenopausal women are shown in Table 1; prevalences for premenopausal women were similar. Women residing in California were more likely to delay childbearing compared with women in other regions, and women in California and the South had slightly fewer children than women in the Northeast and Midwest. Women in California were much more likely to use oral contraceptives and postmenopausal hormones, to have had a mammographic examination, and to consume alcohol than other women. Women in the Midwest and Northeast, in general, were heavier than other women both at age 18 years and currently. Age at menopause varied by region; southern women were slightly younger at menopause than other women. In summary, the group residing in California had the highest prevalence of established breast cancer risk factors and women in the Midwest had the lowest.

Table 1. Age-standardized distribution* of breast cancer risk factors for postmenopausal women by region

	California, %	Northeast, %	Midwest, %	South, %
Menarche, ≤12 y	45.8	47.0	47.0	45.8
Nulliparous	10.1	7.9	7.4	9.5
Parity, ≥5 (among parous women)	15.8	21.2	22.3	13.9
≤24 y at first birth (among parous women)	43.2	49.8	53.7	51.7
≥30 y at first birth (among parous women)	15.5	12.0	10.0	10.3
Ever use of oral contraceptives	37.7	29.3	36.3	31.6
Current use of postmenopausal hormones, ≥5 y	19.9	7.9	13.1	18.1
History of benign breast disease	29.6	26.5	28.8	31.1
Family history in mother or sister	8.8	8.6	8.2	8.3
Height, ≥168 cm	23.8	19.4	20.3	23.6
Body mass index at age 18 y, <19.0	14.5	11.5	12.5	13.8
Body mass index at age 18 y, ≥24.0	12.8	17.0	17.8	14.5
Current body mass index, <21.0	14.6	11.2	11.7	13.4
Current body mass index, ≥29.0	12.1	15.2	16.5	12.8
Alcohol, ≥15 g/day	14.3	10.7	8.0	9.9
Ever mammogram by 1988	83.8	74.8	77.6	77.5
Age at menopause, <40 y	13.4	11.5	11.6	15.9
Age at menopause, ≥50 y	41.3	41.5	40.7	34.9

*Percents represent the age-adjusted person-time allocated to that category divided by the total person-time of follow-up for the region.

Invasive Breast Cancer

Between 1976 and 1992, 3603 incident cases of invasive breast cancer occurred among 118 349 nurses during 1 794 565 person-years of follow-up. The overall age-adjusted incidence rate was 200.8 cases per 100 000 person-years. As defined by residence at baseline in 1976, the region-specific incidence rates (per 100 000 person-years), standardized to the age distribution of the entire cohort, were 225.1 in California, 197.9 in the Northeast, 196.5 in the Midwest, and 193.4 in the South. The overall incidence rate observed in the Nurses' Health Study was 8% higher than the expected incidence rate calculated using the SEER program incidence rates for white women during a similar period (1976 through 1990).

Among all women, region contributed significantly to the age-adjusted model ($P = .05$). We observed a small, but statistically significant, elevation of the age-adjusted breast cancer incidence in California compared with the South (RR = 1.16; 95% CI = 1.02–1.32) (Table 2). However, the incidence rates in the Northeast and the Midwest were not elevated relative to the South. After adjusting for established breast cancer risk factors, the contribution of region to the model was no longer significant. However, the RR for California was only slightly attenuated and still of borderline significance (RR = 1.13; 95% CI = 0.99–1.29). The RRs for the Northeast and the Midwest were similar to the age-adjusted values. Including cases of *in situ* carcinoma of the breast along with invasive breast cancer cases did not notably change the age-adjusted RRs. The RRs for the established breast cancer risk factors were consistent with results from previous reports *(24)*.

During the period of follow-up, 1196 premenopausal women and 2005 postmenopausal women developed invasive breast cancer. For the premenopausal women, there was little evidence of regional variation in either the age-adjusted or multivariate-adjusted analyses (Table 2). For the postmenopausal women, we observed a statistically significant el-

Table 2. Relative risk (RR) of invasive breast cancer incidence in relation to region of residence in the United States, by menopausal status, among 118 349 women aged 30–55 years in 1976 and followed through 1992

	California	Northeast	Midwest	South
		All women*		
No. of cases	535	2034	639	395
Person-years of observation	220 476	1 048 085	329 008	196 996
RR (age adjusted) (95% CI)	1.16 (1.02–1.32)	1.02 (0.92–1.14)	1.02 (0.90–1.15)	1.00
RR (multivariate) (95% CI)†	1.13 (0.99–1.29)	1.05 (0.94–1.17)	1.03 (0.91–1.17)	1.00
		Premenopausal women		
No. of cases	142	717	223	114
Person-years of observation	83 093	489 300	152 206	75 740
RR (age adjusted) (95% CI)	1.07 (0.83–1.36)	0.98 (0.80–1.19)	0.99 (0.79–1.23)	1.00
RR (multivariate) (95% CI)‡	1.02 (0.80–1.31)	1.01 (0.83–1.23)	1.02 (0.81–1.28)	1.00
		Postmenopausal women		
No. of cases	327	1103	353	222
Person-years of observation	103 956	420 610	134 418	90 585
RR (age adjusted) (95% CI)	1.24 (1.05–1.47)	1.08 (0.93–1.24)	1.08 (0.91–1.27)	1.00
RR (multivariate) (95% CI)§	1.18 (1.00–1.40)	1.12 (0.97–1.30)	1.09 (0.92–1.29)	1.00

*Women of uncertain menopausal status were included in analyses of all women but were excluded from the stratified analyses.

†Multivariate RR and 95% confidence interval (CI), adjusted for age in 5-year categories, age at menarche (⩽12, 13, or ⩾14 years), parity (nulliparous, 1–2, 3–4, or ⩾5), age at first birth (nulliparous, ⩽24, 25–29, or ⩾30 years), use of oral contraceptives (ever or never), menopausal status (premenopausal, postmenopausal, or unknown), use and duration of use of postmenopausal hormones (never use, current use <5 years, current use ⩾5 years, or past use), history of breast cancer in a mother or sister, history of benign breast disease, and body mass index (five groups).

‡Multivariate RR and 95% CIs, adjusted for same risk factors as in full cohort analysis, except menopausal status and postmenopausal hormone use, are excluded from models.

§Multivariate RR and 95% CIs, adjusted for same risk factors as full cohort analysis, with the addition of age at menopause in 2-year categories.

evated age-adjusted incidence rate in California (RR = 1.24; 95% CI = 1.05–1.47). The age-adjusted RR in both the Northeast and Midwest was 1.08, and neither was statistically significant. After adjusting for all of the breast cancer risk factors, the excess rate in California was attenuated by 25% (RR = 1.18) but remained of borderline significance (95% CI = 1.00–1.40). The strongest confounding factors were age at first birth, postmenopausal hormone use, and age at menopause. Finer categories of duration of postmenopausal hormone use did not change the association between region and breast cancer risk. Controlling for type of menopause (natural, surgical, or other) also did not alter the association. The increased risk in California was apparent in both the northern and southern halves of the state.

In age-adjusted mortality analyses based on 82 deaths in California and 52 deaths in the South that occurred among postmenopausal women who were cancer free at baseline, risk of breast cancer death was nonsignificantly higher in California, RR = 1.34 (95% CI = 0.95–1.89).

Ninety-four percent of the cohort lived in the same region in 1986 as they did in 1976. When we restricted the cohort to these women, the RRs for invasive breast cancer incidence were comparable to those obtained with the full cohort. Additionally, we restricted the cohort to women who lived in the same region at birth, at age 15 years, at age 30 years, and in 1976. The results were similar to those obtained using the full cohort, except for a stronger association observed for California (Table 3).

Adjusting for alcohol intake for the

Table 3. Age-adjusted relative risk (RR) of invasive breast cancer incidence (95% confidence interval [CI]) for region, restricted to women who lived in the same region throughout their lifetime:* follow-up 1976–1992

	California	Northeast	Midwest	South
		All women†		
No. of cases	92	1123	335	113
Person-years of observation	41 248	658 713	197 572	70 579
RR (age-adjusted) (95% CI)	1.37 (1.04–1.80)	1.10 (0.91–1.34)	1.09 (0.88–1.35)	1.00
RR (multivariate) (95% CI)‡	1.35 (1.02–1.78)	1.14 (0.94–1.39)	1.13 (0.91–1.40)	1.00
		Premenopausal women		
No. of cases	31	408	119	36
Person-years of observation	18 705	331 033	98 348	29 894
RR (age-adjusted) (95% CI)	1.33 (0.82–2.14)	1.00 (0.71–1.40)	1.00 (0.69–1.46)	1.00
RR (multivariate) (95% CI)‡	1.27 (0.79–2.05)	1.04 (0.74–1.47)	1.05 (0.72–1.52)	1.00
		Postmenopausal women		
No. of cases	50	617	186	63
Person-years of observation	17 938	268 672	80 436	31 966
RR (age-adjusted) (95% CI)	1.34 (0.92–1.94)	1.16 (0.89–1.50)	1.17 (0.88–1.55)	1.00
RR (multivariate) (95% CI)‡	1.31 (0.90–1.91)	1.22 (0.94–1.59)	1.19 (0.89–1.59)	1.00

*As defined by living in the same region at birth, age 15 years, and age 30 years. This question was asked in 1992, therefore only women who answered that questionnaire were eligible for this analysis.

†Women of uncertain menopausal status were included in analyses of all women but were excluded from the stratified analyses.

‡Multivariate RR and 95% CIs, adjusted for breast cancer risk factors as described in Table 2.

time period 1980 through 1992 only slightly attenuated the RR comparing California to the South; multivariate RR = 1.19 (95% CI = 0.97–1.46) with alcohol versus RR = 1.21 (95% CI = 0.99–1.48) without alcohol. Adjustment for alcohol did not alter the lack of association between the other regions and the risk of breast cancer. California had the largest percentage of non-Caucasian population (8%). However, restricting the cohort to white women defined as those who did not report Hispanic, African-American, or Asian ancestry did not materially change the RRs. Results were also similar when we restricted the cohort to women who had had at least one mammographic examination.

Discussion

In prospective analyses of a socioeconomically restricted cohort with members drawn from all four U.S. census-defined regions, we did not observe the hypothesized elevated rate of breast cancer incidence in the Northeast compared with the South nor did we see a significant elevation in the Midwest. Premenopausal breast cancer incidence did not vary significantly by region. We observed a marginally statistically significant elevated age-adjusted breast cancer incidence rate in California among postmenopausal women that increased slightly when we restricted the analysis to women who had lived in the same region throughout most of their lives. In this cohort, the South had a slightly higher prevalence of risk factors for breast cancer compared with the Northeast and Midwest for both premenopausal and postmenopausal women and a slightly lower prevalence of risk factors compared with California. After controlling for these factors, 25% of the excess rate of postmenopausal breast cancer in California was explained.

Our results were consistent in direction, although not in magnitude, with previous mortality studies. Sturgeon et al. *(4)* used data from the National Center for Health Statistics from 1987 and observed elevated age-adjusted mortality rate ratios in all regions compared with the South for women aged 50–79 years. The RRs were 1.15 in the West, 1.30 in the Northeast, and 1.18 in the Midwest. In an ecological analysis controlling for group-defined risk and prognostic factors, they were able to explain 50% of the excess mortality in the Northeast and Midwest, but only 10% in the West *(4)*. Blot et al. *(2)* observed a 20% increased rate of breast cancer death in large counties of the Northeast compared with large counties of the South and a 50% increase when they compared small counties. The excess risk in the West ranged from 7% to 30% in large and small counties, respectively. The use of 1960 census data to control for income, urbanization, birth rate, and German or Scandinavian ancestry did not eliminate the region effect among older women *(2)*.

A limitation of our study is that we used only 11 states to make inferences about four large regions. However, these states contain 53% of the entire U.S. population and account for large proportions of the populations of their respective regions *(17)*. With the exception of an under-ascertainment of women in large urban counties in the Northeast and in small counties in the South, the county-specific geographic distribution of participants in the Nurses' Health Study is remarkably representative of these states (Laden F, Neas LM, Hunter DJ: unpublished data). However, these states are not necessarily representative of the region as a whole, particularly in the South and West. For example, the proportion of college-educated persons in Florida, Maryland, and Texas is closer to the proportion observed in the northern states than to the proportion in the remainder of the southern region *(11)*. This limitation may explain why we did not observe RRs for the Northeast and Midwest of the same magnitude as seen in the previous mortality studies. Furthermore, using only California to represent the West may explain why our RR in the West is higher than previously observed. The breast cancer mortality rate in California (1986–1990) was the highest in the Western region and the San Francisco SEER registry reports the highest incidence rate of all registries in the nation *(1)*.

The fact that in most states the geographic distribution of nurses by county was similar to that of white women in general suggests that we did not fail to detect an elevation of risk in certain states due to underrepresentation of individual counties in which breast cancer rates may be higher. We cannot exclude the possibility, however, that very localized exposures within counties might cause breast cancer and be more common in some states than others or that within each county nurses were systematically less likely to live near these sources of exposure. Our results do diminish the likelihood that environmental exposures that are widespread and differ between regions cause large differences in breast cancer rates.

Another potential limitation of this study is that we did not have prospective information on screening. However, the prevalence of mammography was high and similar in all regions, suggesting that differential mammography rates were unlikely to have had substantial influence on the results. Results were similar when we restricted the analysis to women who had had at least one mammographic examination.

Residual confounding could be responsible for our inability to explain some of the excess age-adjusted rate of breast cancer in California compared with the South. We were not able to control for potential risk factors such as physical activity, diet, or alcohol consumption in early life. We did not directly measure hypothesized environmental risk factors for breast cancer, such as reduced sunlight *(25)*, electromagnetic fields *(26,27)*, exposure to organochlorine compounds *(28,29)*, and other pollutants *(30)*. Thus, our results do not rule out the possibility that differences in exposure to these factors between California and the rest of the country might be responsible for some of the small residual difference in breast cancer incidence that was observed. Reassuringly, a recent study showed that regional differences in known breast cancer risk factors completely accounted for the modest elevation in breast cancer incidence rates in the San Francisco Bay Area compared with seven other SEER registries *(31)*.

Use of the Nurses' Health Study cohort restricts the study population to one occupational group of mostly Caucasian women. Thus, the range of possible occupational exposures is reduced, limiting the generalizability of the study. However, this restriction allows us to focus on nonoccupational environmental exposures that might be associated with region. Also, because the Nurses' Health Study is relatively homogeneous com-

pared with the general population, we indirectly controlled for potential confounding by socioeconomic status, and the participants' relatively good access to health care should reduce potential confounding by regional differences in early diagnosis. Aspects of socioeconomic status that vary greatly by region in the general population may explain why we did not see the same magnitude of regional variation reported in previous studies.

Despite these limitations, this study assesses nationwide variation of breast cancer incidence rates in a prospective analysis using risk factors assessed at the individual instead of the group level. The use of incidence, as opposed to mortality rates, avoids bias from potential regional differences in early detection and treatment effectiveness as well as possible differential migration among cases of breast cancer due to health care concerns or retirement. Our results suggest that there is a small excess age-adjusted incidence of postmenopausal breast cancer in California but not in the Northeast or Midwest. Some of the excess rate in California can be explained by established risk factors. Geographic variation in breast cancer rates at the state or regional level is unlikely to be due to region-specific differences in exposures to widespread nonoccupational environmental pollutants.

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Notes

[1]*Editor's note:* SEER is a set of geographically defined, population-based central tumor registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Each registry annually submits its cases to the NCI on a computer tape. These computer tapes are then edited by the NCI and made available for analysis.

Supported in part by Public Health Service grants CA/ES62984 (National Cancer Institute and the National Institute of Environmental Health Sciences) and Institutional National Service Award 5T32CA09001 (F. Laden) and grant CA40356 (National Cancer Institute), National Institutes of Health, Department of Health and Human Services; and by American Cancer Society Faculty Research Award FRA-455 (D. J. Hunter).

We thank the registered nurses who participated in this study, Gary Chase, Karen Corsano, and Barbara Egan.

Manuscript received March 10, 1997; revised June 16, 1997; accepted July 17, 1997.